# Proflora®4R

## (Restorative Probiotic Combination)

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**Active Ingredients:** Proprietary Probiotic Blend (*Bacillus subtilis* DE111®, LactoSpore® (*Bacillus coagulans* MTCC 5856), MuniSpore™ (*Bacillus clausii* CSI08), quercetin, organic marshmallow root (*Althea officinalis*), aloe vera leaf extract (*Aloe barbadensis*).

**Other Ingredients:** Microcrystalline cellulose, vegetable capsule.

### Spore-Forming Probiotics

*Bacillus* species are a group of saprophytic Gram-positive, rod-shaped, spore-forming aerobic or facultatively anaerobic bacteria.[1] They are commonly found in soil, water, dust, air and food products of plant origin. Some strains can also be found in the normal gut microflora.[1,2,3] Bacterial spore formers, in particular those of the *Bacillus* species, exhibit probiotic attributes and have traditionally been used for the production and preservation of food in many different countries.[1] They also have a long-standing use as over-the-counter dietary supplements and registered medicines in certain countries, and more recently are being incorporated into functional foods.[4,5] The genus *Bacillus* is in fact closely related to *Lactobacillus* spp., with both belonging to the phylum *Firmicutes*.[1] Of the hundred species contained in the *Bacillus* genus, only a select few are used as probiotics, including: *Bacillus subtilis, Bacillus coagulans, Bacillus clausii*, *Bacillus pumilus*, *Bacillus licheniformis, Bacillus natto (subtilis), Bacillus polyfermentans*, *Bacillus cereus var. toyoi*, and *Bacillus cereus*.[3,6]

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[1] Elshaghabee FMF, Rokana N, Gulhane RD, et al. Bacillus As Potential Probiotics: Status, Concerns, and Future Perspectives. Front Microbiol. 2017 Aug 10;8:1490.
[2] Hong HA, Duc le H, Cutting SM. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev. 2005 Sep;29(4):813-35.
[3] Schultz M, Burton JP, Chanyi RM. Chapter 11 - Use of Bacillus in Human Intestinal Probiotic Applications. Floch, Martin H. In: Ringel Y, Walker WA, eds. The Microbiota in Gastrointestinal Pathophysiology. Boston: Academic Press; 2017:119123.
[4] Cutting SM. Bacillus probiotics. Food Microbiol. 2011 Apr;28(2):214-20.
[5] Permpoonpattana P, Hong HA, Khaneja R, et al. Evaluation of Bacillus subtilis strains as probiotics and their potential as a food ingredient. Benef Microbes. 2012 Jun 1;3(2):127-35.
[6] Suva MA, Sureja VP, Kheni DB. Novel insight on probiotic Bacillus subtilis: Mechanism of action and clinical applications. Cur Res Sci Med. 2016;2:65-72.

Bacterial endospores offer numerous advantages over conventional non-spore forming probiotics.[1,7] Endospores are dormant life forms and are therefore able survive in desiccated and dehydrated states indefinitely.[7] Due to the ability of *Bacillus* species to form endospores, they remain more stable when processed as foods or in pharmaceutical preparations.[1] Endospores can be stored at room temperature, are easily incorporated into everyday foods, and are stable for several years. They are capable of surviving the low pH of the gastric environment and are resistant to bile salts. The fact that bacterial endospores are able to grow within the intestinal tract, indicates that they are not foreign inhabitants, rather they may exert a symbiotic relationship within the human host.[1,3,4]

*Bacillus* species are capable of sporulation, a complex process resulting in a modified cell that can survive diverse environments including desiccation, ultraviolet radiation, high to extreme cold temperatures, hydrated environments, as well as exposure to solvents, hydrogen peroxide and enzymes.[3] The spore of *Bacillus* is defined as an endospore as it is not a true spore.[3] Bacterial endospores hold a condensed and inactive chromosome at its core.[6] Receptors on the cell's surface are able to detect favorable growth conditions, reversing the process of sporulation so the mother cell can again replicate by binary fission.[3,8]

The mechanism of action by which *Bacillus* species demonstrate health benefits include immune modulation, antioxidant and antimicrobial activities and competitive exclusion of pathogens.[1,2,4] *Bacillus* species are metabolically active and have been shown to produce a number of extracellular enzymes, antimicrobial compounds such as bacteriocins and bacteriocin-like inhibitory substances such as subtilin and coagulin, as well as antibiotic substances (surfactin and bacilysin).[1,3]

Several clinical trials with *Bacillus* probiotics have demonstrated immune-enhancing effects including immune stimulation via the upregulation of secretory immunoglobulin A (sIgA) and increased anti-inflammatory cytokine production. Improvements in gastrointestinal symptoms such as intestinal discomfort, abdominal pain, bloating and diarrhea in various age groups have also been documented. Clinical conditions which have benefited from probiotics containing *Bacillus* species include irritable bowel syndrome (IBS), acute diarrhea, constipation and acquired immunodeficiency syndrome.[3]

## *Bacillus subtilis*

**Biological Actions:**

[7] Casula G, Cutting SM. Bacillus probiotics: spore germination in the gastrointestinal tract. Appl Environ Microbiol. 2002 May;68(5):2344-52.
[8] Setlow P. Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol. 2006 Sep;101(3):514-25.

Antimicrobial, antidiarrheal, immunostimulatory, competitive exclusion of pathogens, prevention of intestinal inflammation, normalization of intestinal flora.[6]

**Traditional Use:**

Members of *Bacillus subtilis* species have traditionally been used in the preparation of fermented foods such as natto in Japan and ogiri in Africa. These fermented foods have a long-standing history of consumption and are believed to be associated with a broad range of health benefits including enhanced immunity and reduced bone loss in post-menopausal women.[9,10]

**Scientific Evidence:**

*Bacillus subtilis* possesses unique characteristics such as endospore formation, versatility of growth nutrients utilization and a high level of enzyme production.[6,11] This particular species of *Bacillus* has a fast growth rate and is able survive in both aerobic and anaerobic conditions.[6] The spores of *Bacillus subtilis* are highly resistant to temperature, extreme pH conditions, gastric acid, bile and solvents, and are therefore able to maintain viability within the gut.[6] For example, in a randomized and double-blinded placebo controlled trial, supplementation with a specific strain, *Bacillus subtilis* DE111®, was shown to survive the gastric environment in healthy humans with ileostomies, allowing the confirmation of both *Bacillus* spores and vegetative (active) forms in the ileum.[12] *Bacillus subtilis* can also be stored for extended periods of time without refrigeration.[6] The therapeutic action of *Bacillus subtilis* spores cover a diverse range of activities such as antimicrobial, antidiarrheal and immunostimulatory properties, competitive exclusion of pathogens, prevention of intestinal inflammation and normalization of intestinal flora.[6]

Beneficial effects of *Bacillus subtilis* supplementation have been reported in several clinical trials. In a double-blinded randomized placebo-controlled trial, healthy participants receiving *Bacillus subtilis* DE111® had an immunomodulating and anti-inflammatory effect on regulatory T cells (Tregs), increasing $CD25^+$ and CD25+FoxP3+ Tregs as well as CD4+CD8+ double-positive T cells in response to stimulation with lipopolysaccharide (LPS, or endotoxin).[13] These changes suggest a mitigation of the inflammatory response induced by low-grade LPS exposure, commonly associated with metabolic

[9] Kojima A, Ikehara S, Kamiya K, et al. Natto Intake is Inversely Associated with Osteoporotic Fracture Risk in Postmenopausal Japanese Women. J Nutr. 2020 Mar 1;150(3):599-605.
[10] Liao C, Ayansola H, Ma Y, et al. Advances in Enhanced Menaquinone-7 Production From Bacillus subtilis. Front Bioeng Biotechnol. 2021 Jul 19;9:695526.
[11] Olmos J, Paniagua-Michel J. Bacillus subtilis A Potential Probiotic Bacterium to Formulate Functional Feeds for Agriculture. J Microb Biochem Technol. 2014;6(7):361-362.
[12] Colom J, Freitas D, Simon A, et al. Presence and Germination of the Probiotic Bacillus subtilis DE111® in the Human Small Intestinal Tract: A Randomized, Crossover, Double-Blind, and Placebo-Controlled Study. Front Microbiol. 2021;12:715863.
[13] Freedman KE, Hill JL, Wei Y, et al. Examining the Gastrointestinal and Immunomodulatory Effects of the Novel Probiotic Bacillus subtilis DE111. Int J Mol Sci. 2021;22(5):2453.

endotoxemia.[14] *Bacillus subtilis* (CU1) supplementation has been shown to stimulate immune function by significantly increasing fecal and salivary sIgA concentrations in the elderly during common infectious disease periods.[15] Supplementation with *Bacillus subtilis* has also been shown to have an anti-inflammatory effect. In an open-label trial, *Bacillus subtilis* HU58 was shown to reduce IL-6 levels by 45% and TNF-α by approximately 55% after 8 weeks of administration to healthy volunteers.[16] Supplementation with *Bacillus subtilis* DE111® was also associated with a reduction in the inflammatory cytokine TNF-α in a controlled clinical trial.[17]

Following oral administration of *Bacillus subtilis* (R0179), *Bacillus* spores were able to transiently colonize the human gastrointestinal tract with low adhesive potential.[18] *Bacillus subtilis* DE111® supplementation was shown to increase the frequency of normal stools among adults over a 10-12 week period compared to controls, and as well as improve symptoms of constipation and diarrhea.[19] Additionally, *Bacillus subtilis* DE111® was shown to reduce the duration of vomiting and hard stools, as well as symptoms of GI discomfort (bloating, diarrhea, constipation) by 62% among children age 2-6 attending daycare, and to also increase the alpha-diversity of the microbiome at the phylum level in this population, with favorable changes including a reduction in the *Firmicutes/Bacteroidetes* ratio compared to placebo.[20,21] Combination therapy with probiotics (*Bacillus subtilis* and *Streptococcus faecium*) and mosapride has been shown to be effective for relief of gastrointestinal symptoms including abdominal pain and discomfort. Supplementation has also demonstrated improved stool frequency and consistency in patients with non-diarrheal-type IBS.[22] In a randomized and double-blind placebo-controlled trial, the combination of *Bacillus subtilis* (MYO2) and *Bacillus coagulans* (MY01) were shown to be both safe and effective in reducing the symptoms of functional dyspepsia, in patients both on and off proton pump inhibitors. Participants receiving this combination also had a decrease in TH17 signaling, as well as increased *Faecalibacterium* in stools, both of which were associated with

[14] Gomes JMG, Costa JA, Alfenas RCG. Metabolic endotoxemia and diabetes mellitus: A systematic review. Metabolism. 2017;68:133-144.
[15] Lefevre M, Racedo SM, Ripert G, et al. Probiotic strain Bacillus subtilis CU1 stimulates immune system of elderly during common infectious disease period: a randomized, double-blind placebo-controlled study. Immun Ageing. 2015 Dec 3;12:24.
[16] Dound YA, Jadhav SS, Devale M, et al. The effect of Probiotic Bacillus subtilis HU58 on Immune function in Healthy Human. The Indian Practitioner. 2017. Sept 70(9):15-20.
[17] Townsend JR, Bender D, Vantrease WC, et al. Effects of Probiotic (Bacillus subtilis DE111) Supplementation on Immune Function, Hormonal Status, and Physical Performance in Division I Baseball Players. Sports (Basel). 2018;6(3):70.
[18] Hanifi A, Culpepper T, Mai V, et al. Evaluation of Bacillus subtilis R0179 on gastrointestinal viability and general wellness: a randomised, double-blind, placebo-controlled trial in healthy adults. Benef Microbes. 2015 Mar;6(1):19-27.
[19] Cuentas, Anamaria et al. The Effect of Bacillus subtilis DE111 on the Daily Bowel Movement Profile for People with Occasional Gastrointestinal Irregularity. Journal of Probiotics & Health. 2017 5: 1-4.
[20] Slivnik M, Kristan KC, Lipovec NC, et al. Effect of Daily Bacillus subtilis DE111® Intake on Gastrointestinal Health and Respiratory Infections in Children Attending Day-care: A Randomised, Parallel, Double-blind, Placebo-controlled Study. J Prob Health. 2020 8:225.
[21] Paytuví-Gallart A, Sanseverino W, Winger AM. Daily intake of probiotic strain Bacillus subtilis DE111 supports a healthy microbiome in children attending day-care. Benef Microbes. 2020;11(7):611-620.
[22] Choi CH, Kwon JG, Kim SK, et al. Efficacy of combination therapy with probiotics and mosapride in patients with IBS without diarrhea: a randomized, double-blind, placebo-controlled, multicenter, phase II trial. Neurogastroenterol Motil. 2015 May;27(5):705-16.

efficacy. Those receiving the combination also had a lower proportion of positive glycocholic acid breath tests, suggesting reduced small intestinal bacterial overgrowth.[23] An excessive TH17/$T_{reg}$ profile has been associated with many chronic inflammatory conditions, and is closely associated with diversity of the microbiome.[24,25]

Another randomized and double-blinded trial found that a 5 species spore-forming probiotic was able to significantly lower triglyceride levels compared to a placebo among participants with mild to moderate hypertriglyceridemia.[26] Although the mechanism is not clearly established, it is likely due to an anti-inflammatory effect, as well as a positive modulation of the microbiome; a previous randomized trial among subjects with post-prandial dietary endotoxemia found that supplementation with a 5 species spore-forming probiotic reduced triglyceride levels by 24%, as well as the level of endotoxemia by 42% (compared to a 36% increase with placebo), with significant reductions in IL-12p70, IL-1β, and ghrelin, indicating a likely improvement in intestinal permeability.[27] Supplementation with *Bacillus subtilis* DE111®, as part of randomized and controlled trial, was associated with a significant reduction in total and non-HDL cholesterol among healthy adult subjects compared to placebo over a 4 week period, with a trend toward improvement in endothelial function (measured via peripheral arterial tonometry, EndoPat).[28] Some evidence suggests *Bacillus subtilis* DE111® may also influence metabolic activity, as supplementation was associated with a reduction in fasting glucose levels among healthy volunteers, as well as a reduction in body fat percentage (compared to placebo) when given during offseason training to division 1 female athletes, as part of an overall training program.[29,30]

In an animal model, *Bacillus* spores were able to stimulate the proliferation of cells within the gut-associated lymphoid tissue and promote a potent immune response.[31] It is believed that following uptake by macrophage cells, *Bacillus* spores interact with lymphocytes and antigen presenting cells in the Peyer's patches to stimulate humoral and cell-mediated immune responses.[31] Administration of

[23] Wauters L, Slaets H, De Paepe K, et al. Efficacy and safety of spore-forming probiotics in the treatment of functional dyspepsia: a pilot randomised, double-blind, placebo-controlled trial. Lancet Gastroenterol Hepatol. 2021 Oct;6(10):784-792.
[24] Essigmann HT, Hoffman KL, Petrosino JF, et al. The impact of the Th17:Treg axis on the IgA-Biome across the glycemic spectrum. PLoS One. 2021 Oct 20;16(10):e0258812.
[25] Pandiyan P, Bhaskaran N, Zou M, et al. Microbiome Dependent Regulation of Tregs and Th17 Cells in Mucosa. Front Immunol. 2019 Mar 8;10:426.
[26] Campbell AW, Sinatra D, Zhang Z, et al. Efficacy of Spore Forming Bacilli Supplementation in Patients with Mild to Moderate Elevation of Triglycerides: A 12 week, Randomized, Double-Blind, Placebo Controlled Trial. Integr Med (Encinitas). 2020 Apr;19(2):22-27.
[27] McFarlin BK, Henning AL, Bowman EM, et al. Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers. World J Gastrointest Pathophysiol. 2017 Aug 15;8(3):117-126.
[28] Trotter RE, Vazquez AR, Grubb DS, et al. Bacillus subtilis DE111 intake may improve blood lipids and endothelial function in healthy adults. Benef Microbes. 2020;11(7):621-630.
[29] Toohey JC, Townsend JR, Johnson SB, et al. Effects of Probiotic (Bacillus subtilis) Supplementation During Offseason Resistance Training in Female Division I Athletes. J Strength Cond Res. 2020;34(11):3173-3181.
[30] Labellarte, G. and Maher, M. (2019) Tolerance and Effect of a Probiotic Supplement Delivered in Capsule Form. Food and Nutrition Sciences, 10, 626-634. Doi:10.4236/fns.2019.106046
[31] Huang JM, La Ragione RM, Nunez A, et al. Immunostimulatory activity of Bacillus spores. FEMS Immunol Med Microbiol. 2008 Jul;53(2):195-203.

*Bacillus subtilis* (A102) has also been shown to enhance the activation of murine macrophages and natural killer cells.[32]

Based on *ex vivo* experiments, the pentapeptide quorum-sensing molecule known as competence and sporulation factor (CSF), excreted by *Bacillus subtilis,* is able to activate key survival pathways in intestinal epithelial cells.[33] CSF also induced cytoprotective heat shock proteins which prevent oxidant-induced intestinal epithelial cell injury and loss of barrier function, thereby helping to maintain intestinal homeostasis.[33] *In vitro* and animal models indicate that CSF simultaneously promotes expression of the anti-inflammatory cytokine IL-10 while inhibiting the production of several inflammatory mediators, reducing intestinal inflammation.[34]

## *Bacillus coagulans*

**Biological Actions:**

Antibacterial, antimicrobial, anti-inflammatory, immunomodulating, antifungal, antidiarrheal, normalization of intestinal flora.[35]

**Traditional Use:**

*Bacillus coagulans* is a spore-forming, microaerophilic, lactic acid producing *Bacillus*. It was first isolated in 1932 and named *Lactobacillus sporogenes*. In 1957 it was reclassified based on its biochemical properties and given the current correct nomenclature of *Bacillus coagulans.* It is considered to be a transient colonizing probiotic, indicating it resides temporarily in the gastrointestinal tract following oral ingestion.[35]

**Scientific Evidence:**

As a spore-forming bacterium, strains of *Bacillus coagulans* are able to survive the acidic environment of the stomach and bile acids and reach the intestine where sporulation occurs.[36] *Bacillus coagulans* lacks the ability to adhere to the intestinal epithelium and is usually eliminated from the intestinal tract within four to five days unless administration is continued.[36] Despite its transient nature, *Bacillus coagulans* is believed to support the intestinal flora and improve the gastrointestinal environment by

[32] Kosaka T, Maeda T, Nakada Y, et al. Effect of Bacillus subtilis spore administration on activation of macrophages and natural killer cells in mice. Vet Microbiol. 1998 Feb 28;60(2-4):215-25.
[33] Fujiya M, Musch MW, Nakagawa Y, et al. The Bacillus subtilis quorum-sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. Cell Host Microbe. 2007 Jun 14;1(4):299-308.
[34] Okamoto K, Fujiya M, Nata T, et al. Competence and sporulation factor derived from Bacillus subtilis improves epithelial cell injury in intestinal inflammation via immunomodulation and cytoprotection. Int J Colorectal Dis. 2012 Aug;27(8):1039-46.
[35] Jurenka JS. Bacillus coagulans: Monograph. Altern Med Rev. 2012 Mar;17(1):76-81.
[36] Endres JR, Clewell A, Jade KA, et al. Safety assessment of a proprietary preparation of a novel Probiotic, Bacillus coagulans, as a food ingredient. Food Chem Toxicol. 2009 Jun;47(6):1231-8.

replenishing desirable microorganisms and antagonizing pathogenic bacteria.[35]

*Bacillus coagulans* produces bacteriocins and bacteriocin-like substances such as coagulin and lactosporin as well as short-chain fatty acids which nourish the colonic mucosa.[35,37] The anti-inflammatory effects of *Bacillus coagulans* include cytokine modulation, inhibition of reactive oxygen species and enhanced phagocytosis. Antifungal activity against *Fusarium* species has also been documented.[35]

Supplementation with *Bacillus coagulans* strains have demonstrated therapeutic benefits in various gastrointestinal conditions across numerous clinical trials. Oral administration of *Bacillus coagulans* MTCC 5856 (LactoSpore®) demonstrated a significant decrease in gastrointestinal symptoms such as bloating, vomiting, diarrhea, abdominal pain, and stool frequency in individuals with diarrhea predominant IBS. Supplementation also led to decreased disease severity and improved quality of life in IBS patients.[38] Additionally, a separate randomized clinical trial conducted among participants with IBS and major depressive disorder found that supplementation with *Bacillus coagulans* MTCC 5856 not only significantly improved IBS symptoms related to quality of life (IBS-QOL), but also led to improvement in several validated scales which assess mood, including HAM-D (Hamilton Rating Scale for Depression), MADRS (Montgomery–Åsberg Depression Rating Scale), CES-D (Centre for Epidemiological Studies–Depression Scale), as well as sleepiness (Modified Epworth Sleepiness Scale, mESS), compared to placebo.[39] While multiple mechanisms are possible for these favorable effects, this trial also demonstrated a reduction in serum levels of myeloperoxidase compared to placebo, a biomarker for oxidative stress and inflammation linked to multiple clinical conditions including depression and cardiovascular disease.[40,41] Several clinical trials have demonstrated both safety and an increased proportion of beneficial bacteria with *Bacillus coagulans* MTCC 5856 supplementation, and *in vitro* data indicates this strain can enhance the production of short-chain fatty acids via fermentation,

[37] Hyronimus B, Le Marrec C, Urdaci MC. Coagulin, a bacteriocin-like inhibitory substance produced by Bacillus coagulans I4. J Appl Microbiol. 1998 Jul;85(1):42-50.
[38] Majeed M, Nagabhushanam K, Natarajan S, et al. Bacillus coagulans MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: a double blind randomized placebo controlled pilot clinical study. Nutr J. 2016 Feb 27;15:21.
[39] Majeed M, Nagabhushanam K, Arumugam S, et al. Bacillus coagulans MTCC 5856 for the management of major depression with irritable bowel syndrome: a randomised, double-blind, placebo controlled, multi-centre, pilot clinical study. Food Nutr Res. 2018;62:10.29219/fnr.v62.1218.
[40] Talarowska M, Bobińska K, Zajączkowska M, et al. Impact of oxidative/nitrosative stress and inflammation on cognitive functions in patients with recurrent depressive disorders. Med Sci Monit. 2014;20:110-115.
[41] Meuwese MC, Stroes ES, Hazen SL, et al. Serum myeloperoxidase levels are associated with the future risk of coronary artery disease in apparently healthy individuals: the EPIC-Norfolk Prospective Population Study. J Am Coll Cardiol. 2007;50(2):159-165.

which may also modulate mood.[42,43,44,45]

Ingestion of *Bacillus coagulans* (GBI-30, 6086) has also been shown to increase beneficial groups of bacteria within the gastrointestinal tract, as well as production of short chain fatty acids, such as butyrate, acetate and propionate.[46,47] Supplementation also resulted in enhanced production of the anti-inflammatory cytokine interleukin (IL)-10, as well as the population of *Faecalibacterium prausnitzii*, an anti-inflammatory commensal bacterium.[47,48] In another clinical trial, *Bacillus coagulans* (GBI-30, 6086) improved the quality of life and reduced gastrointestinal symptoms in adults with post prandial intestinal gas-related symptoms.[49] Administration of this same strain has also been shown to increase the immune response following a viral challenge to adenovirus and influenza A.[50]

Numerous clinical trials suggest a clinically relevant benefit of supplementation with various species of *Bacillus coagulans* for symptom improvement among patients with IBS as observed with MTCC 5856. Administration of *Bacillus coagulans* (Colinox®) in combination with simethicone has been shown to reduce bloating and discomfort in individuals with IBS.[51] Among participants with IBS beginning a low FODMAP diet, those supplemented with both inulin and *Bacillus coagulans* had a significant improvement in symptoms, particularly severity, compared to the FODMAP diet alone.[52] Supplementation with *Bacillus coagulans* (Unique IS2) has been shown to relieve symptoms in both children and adults with IBS, improving abdominal pain, disease severity, and other clinical characteristics.[53,54] This same strain has demonstrated efficacy in clinical trials of constipation as

[42] Majeed, M., Nagabhushanam, K., Mundkur, L., et al. Probiotic modulation of gut microbiota by Bacillus coagulans MTCC 5856 in healthy subjects: A randomized, double-blind, placebo-control study. Medicine. 2023;102(20), e33751.
[43] Burton TC, Lv N, Tsai P, et al. Associations between fecal short-chain fatty acids, plasma inflammatory cytokines, and dietary markers with depression and anxiety: Post hoc analysis of the ENGAGE-2 pilot trial. Am J Clin Nutr. 2023;117(4):717-730.
[44] Majeed M, Majeed S, Nagabhushanam K, et al. Galactomannan from Trigonella foenum-graecum L. seed: Prebiotic application and its fermentation by the probiotic Bacillus coagulans strain MTCC 5856. Food Sci Nutr. 2018;6(3):666-673.
[45] Majeed, M., Nagabhushanam, K., Arumugam, S., et al. Cranberry seed fibre: a promising prebiotic fibre and its fermentation by the probiotic Bacillus coagulans MTCC 5856. Int J Food Sci Technol. 2018; 53:1640-1647.
[46] Nyangale EP, Farmer S, Keller Det al. Effect of prebiotics on the fecal microbiota of elderly volunteers after dietary supplementation of Bacillus coagulans GBI-30, 6086. Anaerobe. 2014 Dec;30:75-81.
[47] Nyangale EP, Farmer S, Cash HA, et al. Bacillus coagulans GBI-30, 6086 Modulates Faecalibacterium prausnitzii in Older Men and Women. J Nutr. 2015 Jul;145(7):1446-52.
[48] Sokol H, Pigneur B, Watterlot L, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008 Oct 28;105(43):16731-6.
[49] Kalman DS, Schwartz HI, Alvarez P, et al. 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. BMC Gastroenterol. 2009 Nov 18;9:85.
[50] Baron M. A patented strain of Bacillus coagulans increased immune response to viral challenge. Postgrad Med. 2009 Mar;121(2):114-8.
[51] Urgesi R, Casale C, Pistelli R, et al. A randomized double-blind placebo-controlled clinical trial on efficacy and safety of association of simethicone and Bacillus coagulans (Colinox®) in patients with irritable bowel syndrome. Eur Rev Med Pharmacol Sci. 2014;18(9):1344-53.
[52] Abhari K, Saadati S, Hosseini-Oskouiee F, et al. Is Bacillus coagulans supplementation plus low FODMAP diet superior to low FODMAP diet in irritable bowel syndrome management? Eur J Nutr. 2020 Aug;59(5):2111-2117.
[53] Madempudi RS, Ahire JJ, Neelamraju J, et al. Randomized clinical trial: the effect of probiotic Bacillus coagulans Unique IS2 vs. placebo on the symptoms management of irritable bowel syndrome in adults. Sci Rep. 2019 Aug 21;9(1):12210.
[54] Sudha MR, et al. Efficacy of Bacillus coagulans Unique IS2 in treatment of irritable bowel syndrome in children: a double blind,

well.[55,56]

Similarly, a randomized trial found that supplementation with *Bacillus* coagulans (SNZ 1969) improved colonic transit time and gut motility among adults with intermittent constipation compared to placebo, effects associated with an increase in *Lactobacillales* and a decrease in *Synergistales* populations.[57] Supplementation with *Bacillus coagulans* (lilac-01) in combination with okra powder has demonstrated significant improvements in bowel movements (defecation frequency) and fecal properties (color, odor and size) in functionally constipated individuals.[58] In a randomized and double-blind clinical trial, *Bacillus coagulans* MTCC 5856 supplementation was shown to dramatically reduce gas, bloating, and flatus related symptoms compared to placebo, when given to participants with functional gas and bloating (but the absence of other GI disorders) as assessed by a gastrointestinal symptom rating scale (GSRS).[59]

*Bacillus coagulans* may also have a beneficial effect on dental caries in children. Rinsing the mouth and swallowing a liquid suspension of *Bacillus coagulans* was found to significantly reduce salivary counts of *Streptococcus mutans*, a bacteria commonly found in the oral cavity associated with tooth decay.[60] Administration via a chewable tablet had comparable effects on saliva levels of the cariogenic species, *Streptococcus mutans* and *Lactobacilli sp.*.[61]

When given along with the prebiotic inulin, supplementation with *Bacillus coagulans* (GBI-30) was shown to significantly reduce liver enzymes, serum tumor necrosis factor-α and nuclear factor-κB activity, as well as hepatic steatosis among participants with non-alcoholic fatty liver disease, compared to placebo.[62]

### *Bacillus clausii*

randomised placebo controlled study. Benef. Microbes. 2018;9:563–572.

[55] Venkataraman R, Shenoy R, Ahire JJ, et al. Effect of Bacillus coagulans Unique IS2 with Lactulose on Functional Constipation in Adults: a Double-Blind Placebo Controlled Study. Probiotics Antimicrob Proteins. 2021 Oct 2.

[56] Madempudi RS, Neelamraju J, Ahire JJ, et al. Bacillus coagulans Unique IS2 in Constipation: A Double-Blind, Placebo-Controlled Study. Probiotics Antimicrob Proteins. 2020 Jun;12(2):335-342.

[57] Kang S, Park MY, Brooks I, et al. Spore-forming Bacillus coagulans SNZ 1969 improved intestinal motility and constipation perception mediated by microbial alterations in healthy adults with mild intermittent constipation: A randomized controlled trial. Food Res Int. 2021 Aug;146:110428.

[58] Minamida K, Nishimura M, Miwa K, et al. Effects of dietary fiber with Bacillus coagulans lilac-01 on bowel movement and fecal properties of healthy volunteers with a tendency for constipation. Biosci Biotechnol Biochem. 2015;79(2):300-6.

[59] Majeed M, Nagabhushanam K, Paulose S, et al. The effects of Bacillus coagulans MTCC 5856 on functional gas and bloating in adults: A randomized, double-blind, placebo-controlled study. Medicine (Baltimore). 2023;102(9):e33109.

[60] Jindal G, Pandey RK, Agarwal J, et al. A comparative evaluation of probiotics on salivary mutans streptococci counts in Indian children. Eur Arch Paediatr Dent. 2011 Aug;12(4):211-5.

[61] Ratna Sudha M, Neelamraju J, Surendra Reddy M, et al. Evaluation of the Effect of Probiotic Bacillus coagulans Unique IS2 on Mutans Streptococci and Lactobacilli Levels in Saliva and Plaque: A Double-Blind, Randomized, Placebo-Controlled Study in Children. Int J Dent. 2020 Dec 29;2020:8891708.

[62] Abhari K, Saadati S, Yari Z, et al. The effects of Bacillus coagulans supplementation in patients with non-alcoholic fatty liver disease: A randomized, placebo-controlled, clinical trial. Clin Nutr ESPEN. 2020 Oct;39:53-60.

**Biological Actions:**

Antimicrobial, antioxidant, immune modulating, gastroprotective, antidiarrheal.[63,64]

**Traditional Use:**

*Bacillus clausii* is a probiotic that has been widely used in Italy for over 40 years for viral diarrhea in children, and for antibiotic-related gastrointestinal side effects.[65]

**Scientific Evidence:**

A spore-forming bacterium, *Bacillus clausii* is able to safely reach the gastrointestinal tract.[63] Human studies infer that orally administered *Bacillus clausii* spores can survive transit through the acidic environment of the stomach and are able to undergo germination, outgrowth and multiplication within the intestines.[66] Due to its chromosomal mutation, *Bacillus clausii* is characterized by an intrinsic resistance to penicillins, cephaolosporins, aminoglycosides, and the macrolides. *B*acillus *clausii* also possesses an acquired resistance to tetracycline, chloramphenicol and to rifampicin, and thus is safe to use in conjunction with antibiotic therapy.[63] *Bacillus clausii* has the ability to modulate the activity of genes linked to inflammatory, immune and defense responses, as well as to intestinal permeability, cell adhesion, cell growth, cell differentiation, cell signaling, apoptosis, signal transcription and transduction.[63,67] *Bacillus clausii* CSI08 (MuniSpore™), for example, has been shown to resist gastric pH and high bile salt concentrations, to attenuate LPS-induced inflammation, and to enhance innate immunity via macrophage stimulation in experimental conditions. This same strain has been shown to have strong antioxidant activity *in vitro*, to express β-galactosidase activity (but not β-glucuronidase), to inhibit the growth of pathogenic bacterial strains, and to likely possess the capacity to synthesize pantothenic acid and cobalamin.[68]

The World Gastroenterology Organization recommends the use of *Bacillus clausii* for antibiotic-associated diarrhea and as an adjunct therapy for *Helicobacter pylori* eradication.[63]

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[63] Lopetuso LR, Scaldaferri F, Franceschi F, et al. Bacillus clausii and gut homeostasis: state of the art and future perspectives. Expert Rev Gastroenterol Hepatol. 2016 Aug;10(8):943-8.
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[68] Khokhlova E, Colom J, Simon A, et al. Immunomodulatory and Antioxidant Properties of a Novel Potential Probiotic Bacillus clausii CSI08. Microorganisms. 2023;11(2):240.

Supplementation with *Bacillus clausii* (UBBC-07) has been shown to be effective in alleviating the symptoms of acute diarrhea (duration, frequency of defecation, abdominal pain, stool consistency) without causing any adverse effects.[69] Supplementation with this same strain has also been associated with improved stool consistency, and both the frequency and duration of diarrhea among children under 5.[70] A systematic review of 6 randomized clinical trials conducted among children with acute diarrhea found supplementation with *Bacillus clausii* (unspecified strains) was effective in reducing the duration of diarrhea as well as the duration of hospitalization compared to placebo (given with oral rehydration salts).[71]

An *in vitro* study revealed that *Bacillus clausii* secretes a protease capable of neutralizing toxins from the toxinogenic *Clostridium difficile* and *Bacillus cereus* strains, possibly explaining its benefit for antibiotic-associated diarrhea.[72] Administration of *Bacillus clausii* has also demonstrated a significant decrease in IL-4 levels, a significant increase in interferon-gamma, transforming growth factor-beta, and IL-10 levels in allergic children and adults.[73,74] A reduction in the duration of recurrent respiratory infections among children supplemented with *Bacillus clausii* was observed in a small randomized trial among children with and without allergies.[75]

In the treatment of small intestinal bacterial overgrowth, supplementation with *Bacillus clausii* over a 1 month period was associated with normalization of the hydrogen glucose breath test in 47% of participants, a comparable rate to a single course of many broad spectrum antibiotics.[76] Supplementation with *Bacillus clausii* (*Enterogermina,* a mix of 4 *Bacillus clausii* strains) has been shown to reduce the incidence and intensity of common side effects associated with *Helicobacter pylori* triple therapy, including nausea, diarrhea and epigastric pain.[65,77] This same mix was shown to reduce the incidence of recurrent calculi-related urinary tract infections when combined with an antibiotic versus an antibiotic alone.[78]

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## Quercetin

**Biological Actions:**

Antioxidant, anti-inflammatory, antiviral, immune-modulating, antihistamine, hepatoprotective, gastroprotective.[79,80,81,82]

**Traditional Use:**

Quercetin is a flavanol which belongs to a group of polyphenolic substances known as flavonoids or bioflavonoids. It can be found in a wide variety of fruits and vegetables such as apples, berries, beans, broccoli and grapes, onions and tomatoes. Quercetin is also naturally present in black tea, green tea, and red wine as well as many seeds and nuts, flowers, bark, and leaves.[80]

**Scientific Evidence:**

Quercetin has several gastrointestinal-related beneficial actions, including antioxidant, anti-inflammatory, anticarcinogenic, antiviral, hepatoprotective and gastroprotective activities. Based on *in vivo* and *in vitro* research, quercetin has the ability to prevent oxidant injury and cell death by scavenging oxygen radicals, protecting against lipid peroxidation and chelating metal ions.[83] The anti-inflammatory activity of quercetin is attributable to its antioxidant activity, including enhancing the expression of antioxidant enzymes (including catalase, superoxide dismutase, and glutathione peroxidase), as well as its inhibitory effects on proinflammatory enzymes (specifically cyclooxygenase and lipoxygenase).[84] It is also involved in the inhibition of inflammatory mediators (leukotrienes and prostaglandins) and helps block histamine release by mast cells and basophils, possibly by binding to the TRPV1 channel.[85,86] Quercetin also has anti-microbial effects via multiple mechanisms including

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therapy in 95 patients with infection calculosis. Chemioterapia. 1985 Dec;4(6):467-70.

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[81] Batiha GE, Beshbishy AM, Ikram M, et al. The Pharmacological Activity, Biochemical Properties, and Pharmacokinetics of the Major Natural Polyphenolic Flavonoid: Quercetin. Foods. 2020 Mar 23;9(3):374.

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destruction of the bacterial cell envelope, preventing bacterial adhesion, inhibiting bacterial nucleic acid synthesis and inhibiting biofilm formation.[87]

Based on *in vitro* research, quercetin has been shown to enhance barrier function in human intestinal Caco-2 cells. The underlying mechanism is thought to be related to the ability of quercetin to promote the assembly of several tight junction proteins such as claudin-1, occludin and zonula occludens-2, and the expression of claudin-4 via the inhibition of protein kinase isoform.[88] Additionally, quercetin is thought to be highly metabolized by gut microbiota, with many active metabolites influencing gastrointestinal and hepatic function. For example, dihydroxyphenylacetic acid, a metabolite of quercetin, has been shown to upregulate transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), preventing acetaminophen associated liver injury in an animal model.[89] Animal models also indicate that quercetin improves diversity of the microbiota following antibiotic treatment, and helps to restore intestinal barrier function, marked by greater mucosal thickness and intestinal villi length.[90]

Research has shown that up to 100 µmol/L luminal quercetin concentration is associated with enhanced intestinal barrier function, which can be achieved by a daily oral intake of as little as 100 mg of quercetin.[88] Supplementation with 50, 100, or 150 mg per day of quercetin has also been found to increase plasma quercetin concentrations by 178% (median change: 92.2 nmol/L), 359% (median change: 171.8 nmol/L), and 570% (median change: 316.2 nmol/L) respectively.[91]

## *Marshmallow (Althea officinalis)*

**Biological Actions:**

Demulcent, mucilage, laxative.[92,93,94]

**Traditional Use:**

Marshmallow has been used in Western herbal medicine to relieve irritation of the oral and pharyngeal

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mucosa due to its demulcent and emollient properties.[95] Traditional indications of marshmallow in herbal medicine include gastritis, peptic ulceration, and inflammation of the gastrointestinal and upper respiratory tracts.

**Scientific Evidence:**

The key active constituents of marshmallow root include polysaccharides (mainly galacturorhamnans, arabinans, glucans and arabinogalactan), tannins, volatile oil, pectin, flavonoids, phenolic acids and asparagine.[93,96] The mucilage content of marshmallow is thought to exert a soothing effect on mucous membranes, in part via the bioadhesion of the polysaccharides to the epithelial mucosa, thereby protecting these cells from irritation, allowing for rehydration of the tissue and providing a shield of the mucosal barrier against physical or microbial stress. Marshmallow extracts and polysaccharide isolates have also demonstrated active stimulatory effects on cell viability and proliferation of human epithelial cells.[96] Marshmallow root also has demonstrated some antimicrobial activity *in vitro*, though to a lesser degree than the positive controls.[97] Marshmallow extracts are widely used to treat various conditions such as coughs, sore throats, various respiratory and gastrointestinal inflammatory conditions, though clinical trial data is lacking.[98]

## *Aloe Vera (Aloe barbadensis)*

**Biological Actions:**

Antifungal, anti-inflammatory, antimicrobial, antioxidant, antiviral, demulcent, emollient, immune enhancing, vulnerary.[79,92,95,99]

**Traditional Use:**

Aloe vera has traditionally been used internally as a general tonic, anti-inflammatory, carminative, laxative, and anti-helminthic agent.[79] In Ayurvedic medicine, aloe gel has been used as a tonic for the liver, spleen, and the blood due to its bitter, astringent, pungent, sweet, and cooling properties.[100]

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[95] Mills S, Bone K. The Essential Guide to Herbal Safety. Philadelphia, U.S.A.: Churchill Livingstone; 2005.
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**Scientific Evidence:**

The key phytochemical compounds of aloe gel include anthraquinones (aloin and emodin), enzymes (catalase, amylase), fatty acids (lupeol and campesterol), polysaccharides (glucomannans) and glycoproteins.[101] Aloe resin, the solid residue obtained from the latex, consists of mainly hydroxyanthracene derivatives.[102] The aloe inner parenchyma, known as the fillet or leaf, also contains 4 tocopherol isoforms (primarily a, but also d, b, and g), as well as mannans, and a diverse variety of polyphenols, antioxidants, and antimicrobial compounds.[103] Aloe vera has been reported to improve parameters of gastrointestinal function including colonic bacterial activity, gastrointestinal pH, stool specific gravity, and gastrointestinal motility.[79] Animal models have shown that aloe polysaccharides improve intestinal permeability, as assessed by the lactulose/mannitol ratio, via an upregulation the tight junction protein zonula occludens (ZO)-1.[104] Animal studies also suggest that glucomannan from aloe vera increases intestinal epithelial cell regeneration via upregulation of the Wnt/β-catenin signaling pathway.[105]

Several clinical trials have reported the beneficial effects of aloe vera administration. Aloe gel has been shown to reduce histological disease activity in patients with ulcerative colitis.[106,107] Supplementation with *Aloe barbadensis* extract (AVH200®) has demonstrated improvement in pain severity, pain frequency and bloating in adult patients with irritable bowel syndrome (IBS).[108] Similarly, two randomized and double-blinded trials found that an aloe inner leaf extract improved symptoms of IBS-D specifically, with significant improvements in abdominal pain severity and frequency.[109] Analysis of

[101] Sánchez M, González-Burgos E, Iglesias I, et al. Pharmacological Update Properties of Aloe Vera and its Major Active Constituents. Molecules. 2020 Mar 13;25(6):1324.
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multiple randomized trials also indicates that aloe may help to improve both glycemic and lipid control, particularly among participants with metabolic abnormalities.[110] Although the mechanisms are unclear, aloe inner leaf gel has been shown to significantly improve the bioavailability of both vitamin C and vitamin B12 in healthy human volunteers when given simultaneously.[111]

Oral administration of aloe vera gel has also been found to reduce the growth of *Candida albicans* in the spleen and kidney (animal research), with *in vitro* data suggesting similar efficacy to standard anti-fungal treatments.[112,113] *In vitro* experiments also show that aloe possesses antimicrobial activity against a number of pathogens including *Helicobacter pylori*, *Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus* (methicillin-resistant strains)*, Escherichia coli, Shigella flexneri, Enterobacter cloacae* and *Enterococcus bovis*, and to inhibit *Staphylococcus aureus* (methicillin resistant) biofilm formation, with components of aloe blocking the initial adhesion and proliferation of biofilms.[114,115,116]

## Proflora®4R Safety Summary:

Multiple strains of each *Bacillus* species found in Proflora®4R have been shown to be safe and well-tolerated in many clinical trials.[6,15,18,22,38,47,49,50,51,58,60,61,65,69,73,74,76,78,117] However, Proflora®4R should be avoided by individuals with an allergy to any of the ingredients, including known hypersensitivities to aloe or quercetin.[118] Adverse effects to quercetin supplementation are rare but may include nausea, dyspnea, headache and mild tingling of the extremities. Animal studies suggest impaired kidney function may be a contraindication to quercetin supplementation, but no human data has confirmed this finding, and it is considered safe and well-tolerated at the recommended dose.[119] Not recommended during pregnancy

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and breastfeeding as safety has not been established for all ingredients during these times.[120,121]

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