

Inhibition of Epsilon Toxin-Producing *Clostridium perfringens* with a Juice-Based Probiotic Medical Food for Dietary Management of Multiple Sclerosis

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Submitted: 2024, Feb 21; Accepted: 2024, Mar 12; Published: 2024, Mar 21

Citation: Kamarei, A. R., Robins, H. F., Finkelstein, E. (2024). Inhibition of Epsilon Toxin-Producing *Clostridium perfringens* with a Juice-Based Probiotic Medical Food for Dietary Management of Multiple Sclerosis. *Int J Prev Med Care*, 2(1), 01-12.

Abstract

Background

Multiple sclerosis (MS) is the most common disabling neurological disease in young adults, with symptom onset generally occurring between the ages of 20 and 40 years. Worldwide, there are 2.8 million people with MS, and in the United States, nearly 1 million. Currently, nine oral medications are FDA-approved for MS, each of which has its own undesirable side effects. The root cause of MS remains unknown, but epsilon toxin-producing *C. perfringens* is implicated as the trigger. Proving this trigger requires the ability to eliminate the bacteria or their toxic effect. As an element of the gut microbiome, epsilon toxin-producing *C. perfringens* may be managed through dietary interventions, but there is no specific dietary supplement or medical food currently available for this purpose.

Materials and Methods

The aim of this *in vitro* research project was to develop a juice-based probiotic medical food for the dietary management of multiple sclerosis by inhibiting the growth of epsilon toxin-producing *C. perfringens*.

For the probiotics, we used a proprietary blend of five strains of *Bifidobacterium* and ten strains of *Lactobacillus* ("Doctor's Biome Signature Probiotic Blend"). For the excipient, we used a proprietary blend of organic green fruit and vegetable juices. The probiotics were added to sterilized excipient at 60 billion colony-forming units per two fluid ounces, yielding a medical food for multiple sclerosis (MF-MS). Two strains of epsilon toxin-producing *C. perfringens*, ATCC 3626 (type B) and ATCC 3631 (type D), were used as the target microorganisms.

Results

Over six days of culture, the control samples (RCM broth) showed drastic population growth of both strains, while the test samples (MF-MS) demonstrated complete inhibition of growth for both strains of epsilon toxin-producing *Closterium perfringens*.

Conclusion

We have developed a patent-pending, juice-based probiotic medical food for the dietary management of MS that inhibits the growth of both type B and type D epsilon toxin-producing *Clostridium perfringens*, which, according to the most recent published clinical findings, are thought to be the cause or trigger of MS. To our knowledge, this is the first *in vitro* study in which such an effect has been clearly demonstrated.

Keywords: Epsilon Toxin-Producing *Clostridium Perfringens*, Multiple Sclerosis, Probiotics, Juice-Based Probiotic Medical Food, Dietary Management Of Multiple Sclerosis.

List of Abbreviations

- ATCC (American Type Culture Collection)
- BDNF (Brain Derived Neurotrophic Factor)
- CID (Chronic Inflammatory Disease)
- CIS (Clinically Isolated Syndrome)
- CFU (Colony Forming Unit)
- CNS (Central Nervous System)
- DBSPB (Doctor's Biome Signature Probiotic Blend)
- DMTs (Disease-Modifying Therapies)
- DMC (Direct Microscopic Count)

- FDA (Food and Drug Organization)
- GBA (Gut-Brain Axis)
- IL-6 (Interleukin 6)
- MF-MS (Medical Food for Multiple Sclerosis)
- MRI (Magnetic Resonance Imaging)
- MS (Multiple Sclerosis)
- NGF (Nerve Growth Factor)
- NMO-SD (Neuromyelitis Optica Spectrum Disorder)
- RCM (Reinforced Clostridial Medium)
- RRMS (Relapsing-Remitting MS)
- PPMS (Primary Progressive MS)
- PYGS (Peptone Yeast Extract Glucose Starch)
- PW (Peptone Water)
- SPMS (Secondary Progressive MS)
- TPGYE (Trypticase-Peptone-Glucose-Yeast-Extract)

1. Background

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is the most common disabling neurological disease in young adults, with symptoms generally appearing between the ages of 20 and 40 years. MS impacts the central nervous system, which is comprised of the brain, spinal cord and optic nerves and controls everything we do [1]. The term “multiple sclerosis” refers to distinctive areas of scar tissue (sclerosis—also called plaques or lesions) that result from the immune system attacking myelin, the protective layer that insulates and allows efficient transmission of signals along the wire-like nerve fibers. Worldwide, 2.8 million people have MS, of which nearly 1 million reside in the U.S. Several governmental organizations, nonprofit organizations, health centers, and companies in the US are actively working to address MS however, no cure for the disease yet exists [2-8].

The exact cause of MS is unknown, but it is established that “something” triggers the immune system to attack the central nervous system. The resulting damage to myelin disrupts signals to and from the brain, causing idiosyncratic and variable symptoms such as numbness, tingling, mood changes, memory problems, pain, fatigue, blindness and/or paralysis. These losses may be temporary or enduring. The particular symptoms experienced depend on the severity of the inflammatory reaction, plaque extent, and plaque location, which can include the brainstem, cerebellum, spinal cord, optic nerves, and white matter around the brain ventricles. No two people have exactly the same symptoms; one might experience only one or two of the possible symptoms, while another may experience several. In addition, each person’s symptoms can change or fluctuate over time. The more common symptoms are fatigue, MS hug, gait difficulties, numbness or tingling, spasticity, weakness, vision problems, vertigo and dizziness, sexual problems, bladder problems, bowel problems and pain and itching [1-2].

At present, there are no symptoms, physical findings or laboratory tests available that can determine if a person has MS. Healthcare providers use several strategies to determine if a person meets the long-established criteria for the diagnosis and to rule out other possible causes of the symptoms being experienced. These strategies include a careful medical history, a neurologic exam

and various tests, including magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) analysis and blood tests.

Currently, multiple sclerosis is classified into four types according to disease course :

- Clinically isolated syndrome (CIS) - The first episode of MS.
- Relapsing-remitting MS (RRMS) - A course of flare-ups (relapse or exacerbation) of new or worsening symptoms followed by periods of remission (when symptoms stabilize or go away); the most common form of MS.
- Primary progressive MS (PPMS) - Symptoms slowly and gradually worsen without any periods of relapse or remission.
- Secondary progressive MS (SPMS) - A diagnosis of RRMS that progresses to SPMS; patients may still experience some relapses or flares, but no longer have periods of remission.

The exact cause of multiple sclerosis is not known, and its pathogenesis is attributed to a combination of factors triggering the disease [2]. Studies support the idea that MS can occur when people with the right combination of genes are exposed to “some trigger” in the environment. This raises the question: what is that “something”? Research suggests that ethnicity and geography play a role. Exposure to certain viruses or bacteria (such as Epstein–Barr virus), where one lives, how the immune system functions, and the genetic variants one carries have all been implicated as contributors to MS pathogenesis. Fully elucidating what causes MS will speed the process of finding more effective ways to treat and cure it. Ideally, we will find a way to prevent MS from developing in the first place.

Current treatments for MS focus on managing symptoms, reducing relapses and slowing disease progression. A comprehensive treatment plan may include the following :

- Disease-modifying therapies (DMTs): Several medications have FDA approval for long-term MS treatment. These drugs help reduce relapses, slow disease progression, and can prevent new lesions from forming on the brain and spinal cord.
- Relapse management medications: For severe attacks, neurologists may recommend a high dose of corticosteroids, which can quickly reduce inflammation and slow damage to the myelin sheath surrounding nerve cells.
- Physical rehabilitation: Multiple sclerosis can affect physical function. Staying physically fit and strong will help maintain mobility.
- Mental health counseling: Coping with a chronic condition can be emotionally challenging; moreover, MS can sometimes affect mood and memory. Working with a neuropsychologist or receiving other emotional support is an essential part of managing the disease.

1.2 Role of Probiotics in Patients with Multiple Sclerosis

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. One of the areas in which probiotics have been investigated during the past decade is neurodegenerative disorders and diseases, reviewed below.

Several studies have investigated the gut-brain axis, defined by Dziedzic and Saluk as the multifactorial interactions between the intestinal microflora and the nervous, immune, and endocrine

systems that connect brain activity and gut functions [9]. Yadav et al. conducted a comprehensive review of the role of the gut-brain axis (GBA) in regulating neurodegenerative diseases and observed disturbance of microbiota, termed dysbiosis, to contribute to conditions such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis; the authors also noted potential possibilities for targeting the gut microbiome to improve neurological health [10]. Alterations in the GBA have been identified in people with MS, suggesting the GBA to have a potential role in disease pathogenesis and therefore promise as a therapeutic target.

Farshbafnadi et al. reviewed studies that examined the relationship of gut microbiota composition to MS and its possible underlying mechanisms [11]. Most such studies agreed that patients with MS suffer from dysbiosis. In addition, altered proportions of certain bacterial phyla were detected in the digestive tracts of these patients compared to healthy individuals. Altieri et al. further described the main changes that occur in the gut microbiota of MS patients, focusing not only on the microbiota and its implications for health and disease, but also the variables that influence it [12]. The authors additionally studied the role of the microbiota as a triggering factor for innate and adaptive immune responses, both in the intestine and in the brain.

Wang et al. reviewed potential mechanisms of gut microbiota involvement in MS pathogenesis, including increasing the permeability of the intestinal barrier, initiating an autoimmune response, disrupting blood-brain barrier integrity, and contributing to chronic inflammation [13]. Zoledziewska reviewed the gut microbiota perspective for interventions in MS and reported the observed impact of the gut microbiome on MS pathophysiology to involve both quantitative and functional changes in composition, metabolism, gut permeability, homeostasis and modulation of the immune system [14]. Ullah et al. likewise reviewed the link between gut dysbiosis and MS development/progression, along with modulation of the gut microbiota as an emerging approach in for prevention and treatment [15]. Shahi et al. extensively studied the role of the gut microbiome in multiple sclerosis, from etiology to therapeutics, and reported it to support diverse physiologic functions, including development and maintenance of the host immune system [16]. Kumar et al. indicated that any change in the gut might increase inflammatory cytokines and affect the quantity of short-chain fatty acids and other metabolites that cause neuroinflammation and demyelination; therefore, alteration of gut microbial composition via probiotic intake may serve as a preventive and treatment strategy [17]. They concluded that it will be easier to develop new therapeutic approaches, particularly probiotic-based supplements, for treating MS if we understand the link between the gut and CNS.

Hashemi et al. reviewed a number of studies using probiotic interventions and determined that probiotics could improve immune cell populations and inflammatory cytokines in patients with MS, potentially contributing to disease management and control [18]. Moravejolahkami et al. conducted a single-center, single-blind randomized clinical trial exploring the effects of

an anti-inflammatory, antioxidant-rich diet and supplemented synbiotic intervention in patients with progressive forms of MS [19]. The results indicated this diet and supplementation to reduce intestinal inflammation and improve clinical manifestations. Jiang et al. performed a systematic review of preclinical trials and a meta-analysis of randomized controlled trials involving MS patients receiving probiotics, for a total of 173 patients. They determined probiotic supplementation to have significant beneficial effects on mental health, and furthermore that it may have beneficial effects on the prevention and treatment of MS [20]. Mirashrafi et al. likewise conducted a systematic review and meta-analysis of clinical trials exploring the effect of probiotic supplementation on disease progression, depression, general health, and anthropometric measurements in relapsing-remitting MS patients [21]. They concluded that probiotic supplementation can improve disease progression and suppress depression and general health in MS patients. Rahimlou et al. conducted a randomized, double-blind, placebo-controlled trial on the effects of long-term administration of multi-strain probiotics on circulating levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and interleukin 6 (IL-6) and mental health in patients with multiple sclerosis and found that probiotic supplementation, compared to placebo, was associated with significant improvements in the general health questionnaire, Beck Depression Inventory-II, Fatigue Severity Scale and Pain Rating Index. However, they did not find any significant difference between the two groups in terms of other factors [22]. Overall, they concluded that six months of probiotic supplementation resulted in improvement of mental health parameters.

Finally, Dunalska et al. conducted a systematic review of current evidence confirming the role of the gut microbiome in the pathophysiology of MS and related disorders, such as neuromyelitis optica spectrum disorder (NMO-SD) [23]. The authors reported the most relevant bacteria for MS pathophysiology to be *Pseudomonas*, *Mycoplasma*, *Haemophilus*, *Blautia*, *Dorea*, *Faecalibacterium*, *Methanobrevibacter*, *Akkermansia* and *Desulfovibrionaceae*, while *Clostridium perfringens* and *Streptococcus* have been demonstrated to play roles in the pathophysiology of NMO-SD.

1.3 Purpose

Currently, there are nine oral medications approved for MS treatment, Aubagio®, Bafiertam®, Gilenya®, Mavenclad®, Mayzent®, Ponvory®, Tecfidera®, Vumerity® and Zeposia® [3]. These are geared towards managing disease symptoms and progression, particularly in relapsing-remitting MS, and each has its own undesirable side effects. No oral medication is available that targets the role of the microbiome in MS pathogenesis. Furthermore, there is presently no dietary supplement or medical food available for specifically targeting epsilon toxin (ETX)-producing strains of *C. perfringens*, which were highlighted in a recent paper by Ma et al. as causative of MS [24].

Published in the *Journal of Clinical Investigation*, Ma et al. (confirming an earlier invention reflected in the US patent number 9,758,573) used quantitative PCR to demonstrate

that people with MS are likely to harbor a greater abundance of epsilon toxin-producing *Clostridium perfringens*, and furthermore demonstrated that epsilon toxin overcomes immune privilege [24]. Based on these findings, *the authors suggest that ETX-producing C. perfringens strains are biologically plausible pathogens involved in triggering inflammatory demyelination in the context of circulating myelin autoreactive lymphocytes.* Finally, they conclude that “Ultimate proof of this hypothesis, as would be the case for any environmental factor, will require a clinical trial to neutralize ETX or to eliminate *C. perfringens* type B or D in the human host.”

We envision that the best approach for validating this hypothesis is to directly address the source of the problem and inhibit growth of the epsilon toxin-producing *Clostridium perfringens* in the gut using a safe and effective probiotic medical food. Our vision is based on the following findings:

- 1) Our first clinical study, in eighty patients with colon disorders, showed the Doctor’s Biome Colon Health (DBCH) dietary supplement (comprising at time of manufacturing 27 billion CFUs of a proprietary probiotic blend [DBSPB] in 2 oz of vegetable and fruit juice) to be highly effective and safe, helping relieve occasional diarrhea, gas and bloating and reducing the risk of colon microbial infections while replenishing the healthy colon microbiome [25].
- 2) Our second in vitro study using the same DBCH dietary supplement revealed DBSPB to release some bioactive compounds (metabolites) into the juice that completely inhibit the growth of *C. difficile* [26].

Now, connecting our findings with the above-reported conclusions of Ma et al., we hypothesized that if we increase the dosage of DBSPB (to 60 billion CFUs at time of manufacturing in 2 oz juice), we can develop a safe and effective probiotic medical

food for the dietary management of MS under the supervision of a physician. This probiotic medical food is expected to have both a general effect (ameliorating dysbiosis) and a specific effect (inhibiting growth of the epsilon toxin-producing *Clostridium perfringens*). We developed such a probiotic medical food and applied for patent protection.

Before embarking on any clinical trial with the developed probiotic medical food for multiple sclerosis, it is necessary to demonstrate that it indeed inhibits the growth of epsilon toxin-producing *C. perfringens* under in vitro conditions. This is the purpose of the research presented in this paper.

2. Materials and Methods

2.1 Probiotics

Bacteria of the genera *Bifidobacterium* and *Lactobacillus* are broadly recognized for their key roles in the human intestinal microflora. We designed a proprietary blend of five strains of *Bifidobacterium* and ten strains of *Lactobacillus* (“Doctor’s Biome Signature Probiotics Blend” = “DBSPB”) obtained from the reputable company Cultures Supporting Life (CSL-USA) (Table 1). Species identification of these probiotics was performed by sequence analysis of the 16S rRNA gene, and strain identification by pulse field gel electrophoresis. These probiotics have been shown to be sensitive to antibiotics and have passed microbiological assays and heavy metal analyses. They are not genetically modified, are free from allergens, are considered safe with respect to bovine spongiform encephalopathy, and do not contain colorants. These probiotics have also been subjected to a series of in vitro tests to assess their gastrointestinal survival (tolerance to hydrochloric acid), tolerance to bile salts, and resistance to gastrointestinal tract enzymes (pepsin and pancreatin).

Doctor's Biome Signature Probiotics Blend (DBSPB)	
Genus/Species	Strain
<i>Bifidobacterium bifidum</i>	SP 9
<i>Bifidobacterium breve</i>	BBR8
<i>Bifidobacterium infantis</i>	SP 37
<i>Bifidobacterium longum</i>	SP 54
<i>Bifidobacterium animalis subsp. lactis</i>	BLC1
<i>Lactobacillus acidophilus</i>	LA1
<i>Lactobacillus brevis</i>	SP 48
<i>Lactobacillus bulgaricus</i>	LB2
<i>Lactobacillus casei</i>	BGP 93
<i>Lactobacillus gasseri</i>	LG050
<i>Lactobacillus paracasei</i>	101/37
<i>Lactobacillus plantarum</i>	14D
<i>Lactobacillus reuteri</i>	LR92
<i>Lactobacillus rhamnosus</i>	SP 1
<i>Lactobacillus salivarius</i>	SP2

Table 1: Doctor's Biome Signature Probiotics Blend (DBSPB) of bifidobacteria and lactobacilli used in the production of Medical Food for Multiple Sclerosis (MF-MS).

As an optimum liquid excipient, we chose a proprietary sterilized diluted blend of 100% organic green fruit and vegetable juices, specifically mint, cucumber, apple, lettuce, kale, celery and lemon juice.

2.2 Product (Probiotic Medical Food for Multiple Sclerosis)

Doctor's Biome Signature Probiotic Blend (DBSPB) was added to the excipient (60 billion CFU per 2 fluid ounces) to produce Probiotic Medical Food for Multiple Sclerosis (MF-MS). The formulation and method of use of this product are protected by a pending patent.

Two-ounce bottles of Doctor's Biome Probiotic Medical Food for Multiple Sclerosis (MF-MS) were delivered to Eurofins Microbiology Laboratories for this study. The samples were stored at refrigeration temperature upon arrival.

2.3 Epsilon Toxin (ETX)-Producing *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, bacillus (rod-shaped), anaerobic, spore-forming pathogenic bacterium that is ever-present in nature and can be found as a normal component of the human intestinal microbiome [27]. In addition, *C. perfringens* is among the most common causes of food poisoning, estimated by the Centers for Disease Control and Prevention to cause nearly 1 million foodborne illnesses in the United States every year. Most people with *C. perfringens* food poisoning have diarrhea and stomach cramps, but no vomiting. Symptoms usually begin 6 to 24 hours after ingesting the bacteria, can start

suddenly, and typically last for less than 24 hours. *C. perfringens* food poisoning is diagnosed when a laboratory test detects the bacteria or associated toxins in a patient's stool sample, or when the bacteria are found in food linked to the illness. Most people recover without antibiotics [28].

McDonel previously characterized the pathogenicity of *C. perfringens*, and reported it to produce at least 12 different antigens, referred to as *toxins*, that may be involved in *pathogenesis* [29]. These antigens are named *alpha*, *beta*, *epsilon*, and *iota* toxin (major toxins); *delta*, *theta*, and *kappa* (collagenase); *lambda* (protease); *mu* (hyaluronidase); *nu* (deoxyribonuclease); and *gamma* and *eta* toxin (minor toxins). Certain strains also produce an *enterotoxin* and a neuraminidase; according to Songer as many as 17 exotoxins of *C. perfringens* have been described in the literature, while Johnston more recently reported a tally of at least 20⁰C [30,31]. *perfringens* strains are currently categorized into seven toxinotypes (A, B, C, D, E, F, and G) based on the presence or absence of six typing toxins (α , β , epsilon, iota, enterotoxin, and netB). Five toxin types (A, B, C, D and E) are definable based upon the production of one or more major protein toxins, as listed in Table 2 [32]. To date, significant progress has been made in characterizing the alpha and theta toxins and enterotoxins, but relatively little is yet known about the other toxins. A variety of disease syndromes are believed to be caused by one or more of these toxins, although their exact role in disease is unclear in most cases.

Toxin	C. perfringens Type					Cellular Target (mode of action)
	A	B	C	D	E	
Alpha	+	+	+	+	+	Membrane (phospholipid destruction)
Beta		+	+			Membrane (pore formation)
Epsilon		+		+		Membrane (pore formation)
Iota					+	Actin (cytoskeleton destruction)

Table 2: Major toxins for *C. perfringens* typing - from Stiles et al. (35)

The Illinois Department of Public Health has published an overview of *C. perfringens* toxins indicating that they can be used as weapons. The toxins can be purified into a concentrated form, and would most likely be aerosolized, although they could also be placed in water or food [33]. Such purified toxins may have multiple effects depending on the strain of bacteria used, the type of toxin purified, the method of release and the amount taken into the body. In particular, *C. perfringens* toxins can cause stomach effects (loss of appetite, nausea, vomiting, and watery or bloody diarrhea with crampy stomach pain) and respiratory effects (difficulty breathing, wheezing and coughing). Mouth and throat pain accompanied by blood in the saliva and sputum may be possible, as may skin effects (burning pain, redness, itching, rash or blisters). Epsilon toxin (ETX) specifically may have brain and nerve effects, as such damage has been demonstrated in laboratory tests on animals, resulting in dizziness, difficulty with balance and coma.

The Arizona Department of Health Services has likewise specifically highlighted the potential of ETX as a potential

biological weapon [34]. This toxin is produced by type B and type D strains of *C. perfringens* and acts to damage cell walls, causing potassium and fluid leakage from cells. ETX can be detected by several assays, including enzyme-linked immunosorbent assays (ELISAs). Willis et al. studied ETX from *C. perfringens* in the context of strain genotype, phenotype and toxin-sensitive cell lines. The American Type Culture Collection (ATCC) *Clostridium perfringens* collection contains representatives from 4 of the 5 toxin types as determined by genotypic and phenotypic methods; strains 3626, 17865, 17870, and 3631 were determined to express ETX specifically [35].

There is no specific treatment or established cure for *C. perfringens* toxins; supportive care (intravenous fluids and medicine to control fever and pain) is the standard treatment. As noted by Stiles et al. ETX has been studied by various groups and is a primary veterinary concern for some large animals; vaccines of varying quality are available for veterinary use, solely to combat epsilon enterotoxemia [32]. There is no vaccine available for humans. Toxin-specific immunoglobulins might

be helpful as a therapeutic or prophylactic agent for humans following a nefarious application of ETX. All told, there is clearly much more to learn about ETX, how it works and how to protect against it.

2.4 Preparation of Microorganisms

Two strains of ETX-producing *Clostridium perfringens*, ATCC 3626 (Type B) and ATCC 3631 (Type D), were purchased from the American Type Culture Collection and used in this study. To cultivate *C. perfringens*, isolated colonies of each strain were transferred to heat-exhausted peptone yeast extract glucose starch (PYGS) broth and incubated anaerobically at 30 °C/86 °F for 3 to 4 days. Afterwards, the grown colonies were transferred to fresh PYGS broth and again incubated anaerobically at 30°C/86°F for 3 to 4 days. Each strain was pelleted by centrifugation, washed once with 0.1% peptone water (PW), and then resuspended in PW to form 2 separate inoculums. The concentration of each inoculum was determined using the direct microscopic count (DMC) method with a Petroff-Hausser counting chamber and

then adjusted to the appropriate concentration using PW. Each final concentration was verified by enumerating appropriate serial dilutions on trypticase-peptone-glucose-yeast extract (TPGYE) agar. Before the study, the cultures were stored in an anaerobic chamber overnight at 4 °C/39 °F, then removed from cold storage and allowed to reach ambient temperature prior to use.

2.5 Preparation of the Test Product and Control Samples

Individual 2-ounce bottles of MF-MS were composited and homogenized to prepare one sample. The combined sample was then centrifuged at 4500 RPM for 15 minutes. After centrifugation, the supernatant was filtered through a 0.45 µm filter to prepare the “test product”. Representative images of the MF-MS before and after centrifugation are shown in Figure 1. The pH of the test product was measured prior to inoculation (one replicate sample). Control samples were prepared using Reinforced Clostridial Medium (RCM) broth.

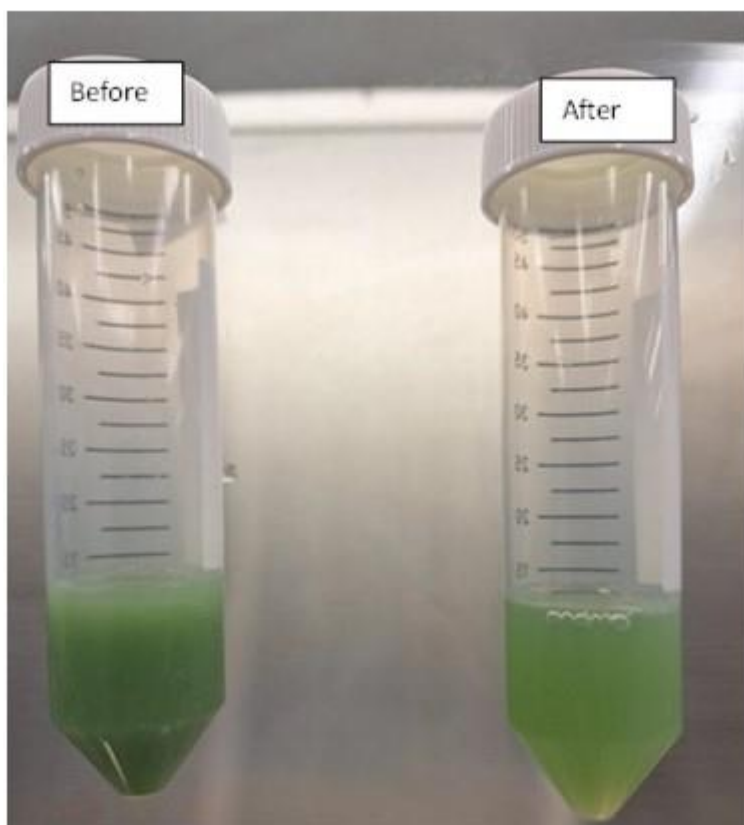


Figure 1: Representative photographs of Doctor’s Biome MF-MS before and after centrifuging at 4500 RPM for 15 minutes.

2.6 Inoculation of Test Samples and Control Samples

After filtration, the product was aseptically dispensed into glass tubes at 2 ml per tube. Each tube was inoculated with one inoculum (either *C. perfringens* ATCC 3626 or ATCC 3631) at a target concentration of ~4 log CFU/ml. The corresponding

control samples (RCM broth) were likewise dispensed into glass tubes at 2 ml per tube and inoculated at a target concentration of ~4 log CFU/ml. Immediately following inoculation, the solutions were mixed to ensure even distribution of the inoculum.

The inoculated test and control samples are summarized below.

- ✓ Test Sample: MF-MS centrifuged and inoculated with *C. perfringens* ATCC 3626
- ✓ Test Sample: MF-MS centrifuged and inoculated with *C. perfringens* ATCC 3631
- ✓ Control Sample: RCM broth inoculated with *C. perfringens* ATCC 3626
- ✓ Control Sample: RCM broth inoculated with *C. perfringens* ATCC 3631

2.7 Uninoculated Negative Control Samples

Additional 2 mL aliquots of the prepared test product were not inoculated with any microorganisms and served as negative controls.

2.8 Incubation of Test Samples and Control Samples

The inoculated test samples and control samples were incubated along with the uninoculated negative control samples under anaerobic conditions at 37 °C/99 °F for up to 6 days. Samples were evaluated according to the time course and replication scheme outlined in Table 3.

Sample Description	Incubation Conditions	Sampling Times	Replicates/Sampling Time
Test Samples	37 °C/99 °F, anaerobic	Days 01, 1, 2, 3, and 6	3 replicates per organism
Control Samples	37 °C/99 °F, anaerobic	Days 01, 1, 2, 3, and 6	3 replicates per organism
Uninoculated Samples	37 °C/99 °F, anaerobic	Days 01, 1, 2, 3, and 6	1 replicate

¹Initial counts of organisms in test or control samples prior to incubation.

Table 3: Sampling scheme for the study

2.9 Evaluation of Test Samples and Control Samples

At each sampling time, three replicate samples per strain were removed from the incubator. Each sample was diluted with PW, plated onto TPGYE agar, and incubated anaerobically for

72 hours at 37 °C/99 °F. Subsequently, colonies were counted based on the characteristic colony morphology typical of *C. perfringens* on TPGYE agar. Representative photographs of one agar plate per sample are provided in Figures 2 and 3.

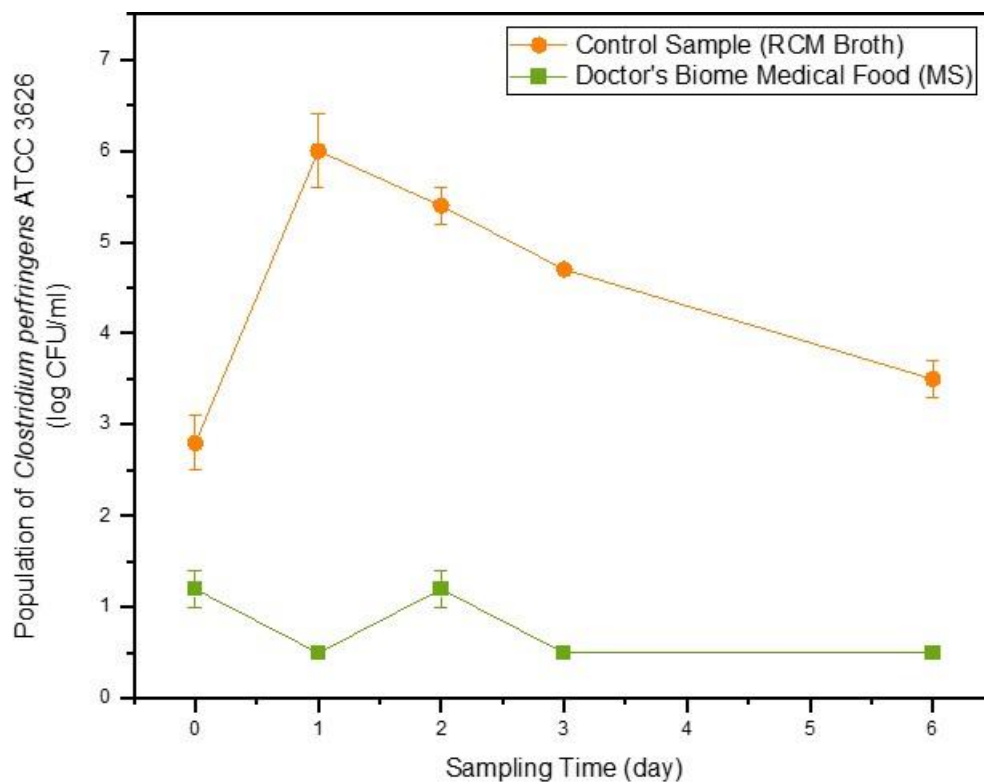


Figure 2: Population and standard deviation of epsilon toxin (ETX)-producing *Clostridium perfringens* ATCC 3626 recovered from inoculated Doctor's Biome Medical Food for MS (test sample) and RCM broth (control sample) incubated anaerobically at 37 °C/99 °F.

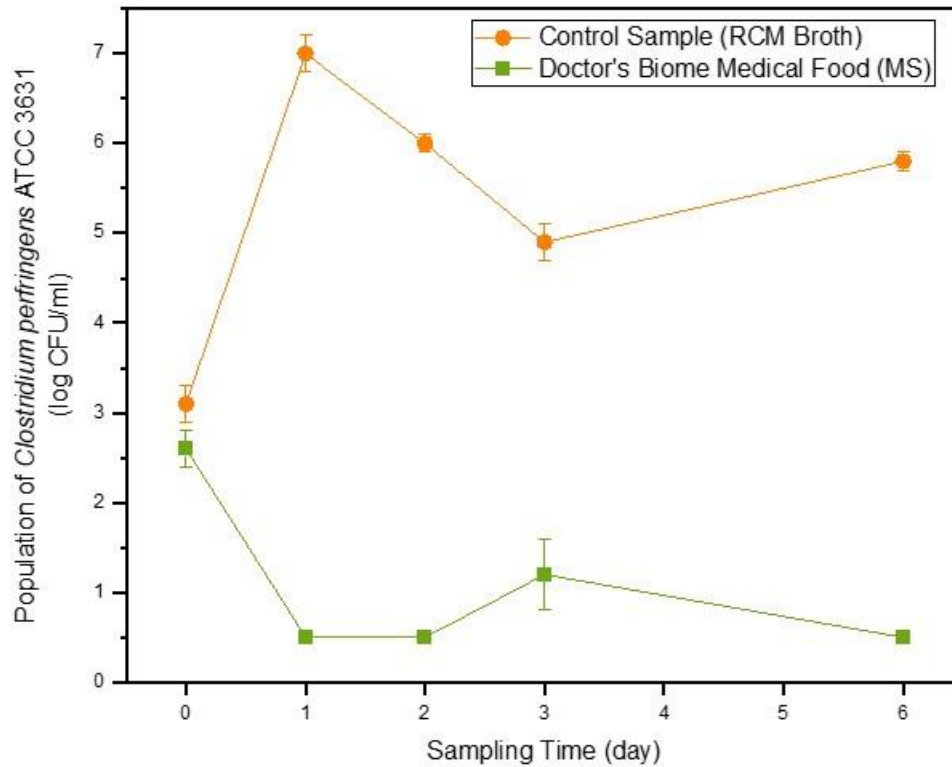


Figure 3: Population and standard deviation of epsilon toxin (ETX)-producing *Clostridium perfringens* ATCC 3631 recovered from inoculated Doctor's Biome Medical Food for MS (test sample) and RCM broth (control sample) incubated anaerobically at 37 °C/99 °F.

2.10 Evaluation of Uninoculated Samples

At each sampling time, one uninoculated sample was removed from the incubator, plated onto TPGYE agar, and incubated anaerobically for 72 hours at 37 °C/99 °F. The pH of the uninoculated sample was also determined at each sampling time.

3. Results

All microbiological data are reported as log CFU/ml. The limit of detection via the plating method was 1.0 log CFU/ml. Table 4 presents the population sizes of *C. perfringens* ATCC 3626 and ATCC 3631 that were recovered from the artificially inoculated test and control samples. Similarly, Table 5 presents the quantities of organisms recovered from uninoculated Doctor's Biome MF-MS samples, along with the measured pH values.

Sampling Time	Replicate	Population Level of <i>C. perfringens</i> ATCC 3626 (log CFU/ml)		Population Level of <i>C. perfringens</i> ATCC 3631 (log CFU/ml)	
		Doctor's Biome MF-MS	Control (RCM)	Doctor's Biome MF-MS	Control (RCM)
Day 0	A	1.3	3.1	2.8	3.2
	B	1.3	2.5	2.7	2.8
	C	<1.0 ²	2.9	2.4	3.1
	Avg ± SD¹	1.2 ± 0.2	2.8 ± 0.3	2.6 ± 0.2	3.1 ± 0.2
Day 1	A	<1.0 ²	5.8	<1.0 ²	7.2
	B	1.0	5.7	<1.0 ²	7.0
	C	<1.0 ²	6.5	<1.0 ²	6.8
	Avg ± SD¹	<1.0	6.0 ± 0.4	<1.0	7.0 ± 0.2
Day 2	A	<1.0 ²	5.6	<1.0 ²	6.0
	B	1.3	5.4	<1.0 ²	6.0
	C	<1.0 ²	5.2	<1.0 ²	5.9
	Avg ± SD¹	1.2 ± 0.2	5.4 ± 0.2	<1.0	6.0 ± 0.1

Day 3	A	<1.0 ²	NA ³	<1.0 ²	4.8
	B	<1.0 ²	4.5	1.7	5.1
	C	<1.0 ²	5.0	<1.0 ²	5.0
	Avg ± SD¹	<1.0	4.7 ± 0.3	1.2 ± 0.4	4.9 ± 0.2
Day 6	A	<1.0 ²	3.6	<1.0 ²	5.9
	B	<1.0 ²	3.3	<1.0 ²	5.8
	C	<1.0 ²	3.6	<1.0 ²	5.7
	Avg ± SD¹	<1.0	3.5 ± 0.2	<1.0	5.8 ± 0.1

¹Avg ± SD: average ± standard deviation;

²Result was below the limit of detection of 1.0 log CFU/ml and was treated as 1.0 for calculations;

³Not applicable due to lab error.

Table 4: Population sizes of *C. perfringens* ATCC 3626 and ATCC 3631 recovered from artificially-inoculated Doctor's Biome MF-MS (test sample) and RCM broth (control sample) incubated anaerobically at 37 °C/99 °F.

Sampling Time	Replicate	Organisms Recovered onto TPGYE Agar (log CFU/ml) ¹	pH
Day 0	A	<1.0 ²	3.10
Day 1	A	<1.0 ²	3.09
Day 2	A	<1.0 ²	3.07
Day 4	A	<1.0 ²	3.09
Day 6	A	<1.0 ²	3.09

¹TPGYE: Trypticase-Peptide-Glucose-Yeast Extract agar;

²Below the limit of detection of 1.0 log CFU/ml.

Table 5: Organisms recovered from uninoculated Doctor's Biome MF-MS samples incubated anaerobically at 37 °C/99 °F.

For *C. perfringens* ATCC 3626, the day 0 population in MF-MS was markedly less than the control (Table 4 and Figure 2). However, growth was completely inhibited in MF-MS throughout the 6-day study period. In contrast, the control sample (RCM) showed a typical growth curve for microorganisms, with a drastic population increase peaking on day 1 and 2, i.e., log phase, followed by a gradual decrease.

For *C. perfringens* ATCC 3631, the day 0 population in MF-MS was almost equal to that in the control sample, but became drastically reduced on day 1, and then had its growth completely inhibited. The control sample conversely exhibited dramatic growth.

Representative photographs of TPGYE agar plates are shown in Figures 4 and 5.

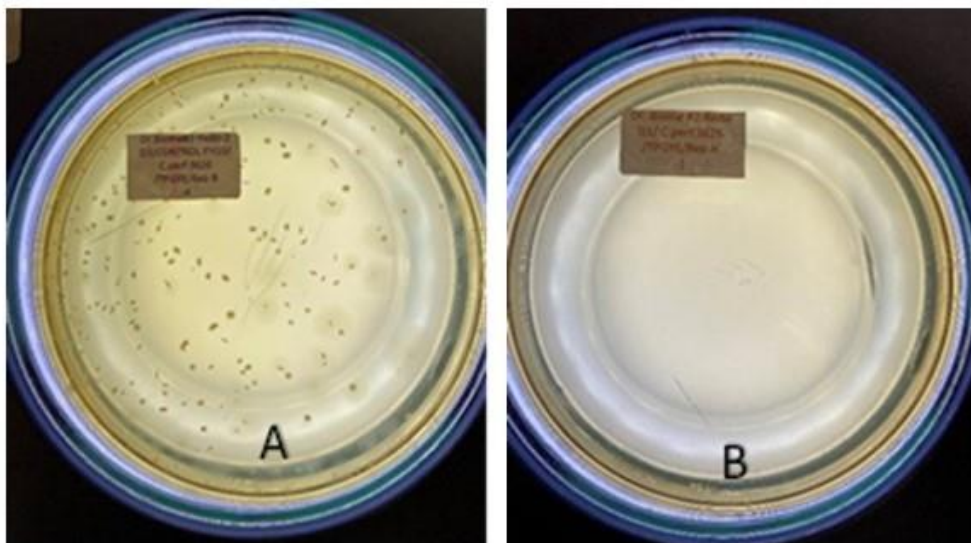


Figure 4: Photos of *C. perfringens* ATCC 3626
 (A) Recovered from the control sample (1:10,000 dilution).
 (B) Recovered from the Doctor's Biome MF-MS sample (1:10 dilution).

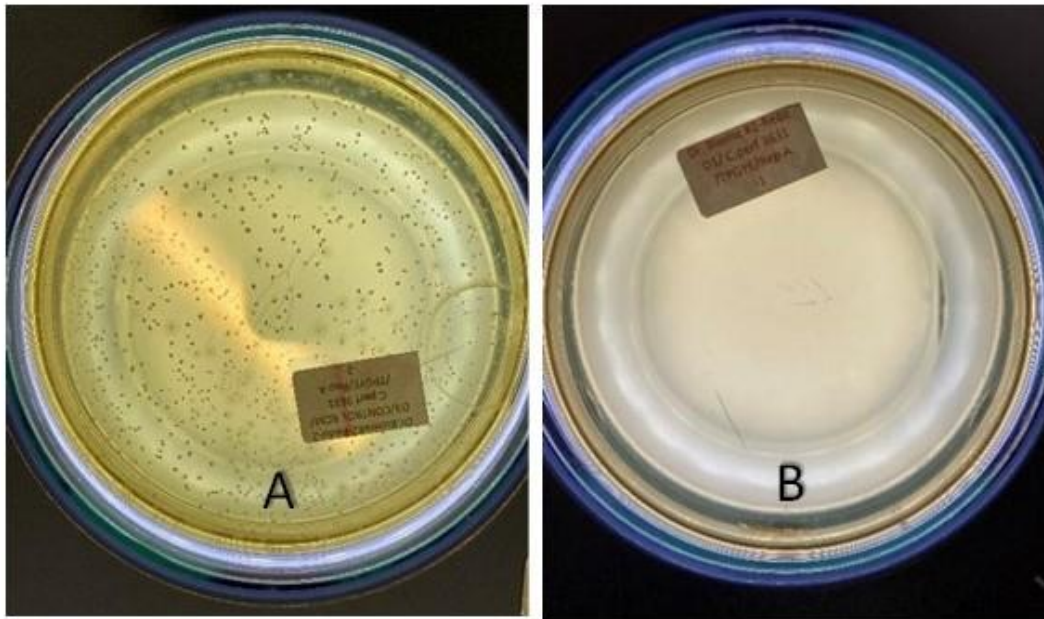


Figure 5: Photos of *C. perfringens* ATCC 3631
 (A) Recovered from the control sample (1:100 dilution).
 (B) Recovered from the Doctor's Biome MF-MS sample (1:10 dilution).

Colonies of *C. perfringens* ATCC 3626 recovered from the control sample at a 1:10,000 dilution were readily evident, while no colonies could be recovered from the test sample even at 1:10 dilution. This indicates total suppression of *C. perfringens* ATCC 3626 by MF-MS.

Similarly, an overwhelming number of *C. perfringens* ATCC 3631 colonies were recovered from the control sample at a 1:100 dilution, while none could be recovered from the test sample even at 1:10 dilution. Thus, MF-MS also totally suppresses *C. perfringens* ATCC 3631.

It seems reasonable to infer that for this total inhibition of *C. perfringens* ATCC 3626 and ATCC 3631, the chosen proprietary blend of Bifidobacterium and Lactobacillus functions in a complementary, additive, or possibly synergistic manner to release bioactive compounds (i.e., postbiotics) into the blend of organic green juices. One such compound could be lactic acid (and possibly other organic acids), which can reduce the pH of the environment to a degree unfavorable for the tested *C. perfringens* strains.

4. Discussion

In terms of efficacy, most probiotics on the market are in the form of capsules (loose powder) or tablets (compressed powder). In these dry forms, probiotic cells are in a state of “suspended animation,” with most of their vital functions temporarily ceased. In other words, while alive at the time of manufacturing and after freeze-drying, these cells are not living (not physiologically active) when consumed. For such cells to become physiologically active, they need to be fully hydrated and immersed in an aqueous environment with sufficient available water and nutrients. Probiotics that are consumed in the form of a dry powder become fully hydrated in the highly acidic environment of the stomach (pH ~ 1.5), which is not ideal

for rehydration; indeed, it is not clear what percentage of dry probiotics can successfully become rehydrated in that context. This suboptimal delivery has led to mixed results in studies investigating the benefits of probiotics under various conditions. However, when probiotics are consumed in liquid form (e.g., in a blend of organic fruit and vegetable juices), they are both fully hydrated and suspended in an aqueous nutritive environment. In other words, such pre-hydrated probiotics are physiologically active and functional upon consumption.

To our knowledge, this is the first study in which a blend of proprietary pre-hydrated probiotics in a proprietary liquid juice medium has completely inhibited epsilon toxin-producing *C. perfringens*. This necessitates further exploration of MF-MS in a clinical setting.

5. Conclusion

Based on the results of this study, we conclude that Doctor's Biome Probiotic Medical Food for Multiple Sclerosis (MF-MS) inhibited the growth of epsilon toxin-producing *C. perfringens* ATCC 3626 and ATCC 3631 in artificially inoculated products during a 6-day period of anaerobic incubation at 37 °C/99 °F. Specifically, the population of *C. perfringens* decreased to <1.0 log CFU/ml after 1 day of incubation and remained either below the limit of detection or slightly above 1.0 log CFU/ml throughout the remainder of the study. In contrast, culture in reinforced clostridial medium broth under the same storage conditions produced exponential growth of both tested strains. In light of these in vitro results, a prospective, randomized, double-blind clinical trial (based on good clinical practice) is warranted to evaluate the safety and efficacy of MF-MS in patients with multiple sclerosis.

All told, Doctor Biome has developed a patent-pending, juice-based probiotic medical food for the dietary management of

multiple sclerosis that has been demonstrated by Eurofins Scientific (a world leader in food and pharma testing) in the laboratory to inhibit the growth of both type B and type D epsilon toxin-producing *Clostridium perfringens*, strains that, according to the most recent published clinical findings, are thought to cause or trigger multiple sclerosis.

Declaration

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed with the results and conclusions of this article and agreed on the journal to which the article will be submitted. All authors reviewed and agreed on the manuscript. All authors agreed to take responsibility and be accountable for the article's contents.

Availability of data and materials

The dataset (final report from Eurofins Microbiology Laboratories) used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

Competing interests

There is no competing interest in this study.

Author contributions

ARK designed and supervised development of the probiotic medical food for multiple sclerosis (MF-MS); proposed and designed the microbiological study and study variables (selection of target organisms, inoculation level, incubation temperature and incubation times); and prepared the first draft of the manuscript. HFR provided some literature and reviewed the draft of the manuscript. EF assisted in the preparation of the product samples, contractual arrangements with Eurofins, and the conversion of tabulated values to graphs, and reviewed the draft of the manuscript.

Acknowledgments

The authors acknowledge the valuable contribution of Fei Wang, Ph.D. (project microbiologist) from the Eurofins Microbiology Laboratories (www.eurofins.com) in performing this inoculation study.

References

1. National Multiple Sclerosis Society (<https://www.nationalmssociety.org/>)
2. National Institute of Neurological Disorders and Stroke (<https://www.ninds.nih.gov/health-information/disorders/multiple-sclerosis>)
3. Multiple Sclerosis Association of America (<https://mymsaa.org/>)
4. Multiple Sclerosis Foundation (<https://msfocus.org/Home.aspx>)
5. Accelerating research toward a cure for multiple sclerosis (<https://www.acceleratedcure.org/>)
6. Mayo Clinic <https://www.mayoclinic.org/diseases-conditions/multiple-sclerosis/symptoms-causes/syc-20350269>
7. Cleveland Clinic <https://my.clevelandclinic.org/health/diseases/17248-multiple-sclerosis>
8. Mount Sinai <https://www.mountsinai.org/health-library/diseases-conditions/multiple-sclerosis>
9. Dziedzic, A., & Saluk, J. (2022). Probiotics and commensal gut microbiota as the effective alternative therapy for multiple sclerosis patients treatment. *International Journal of Molecular Sciences*, 23(22), 14478.
10. Yadav, H., Bhardwaj, R., Bakshi, A., Gupta, S., & Maurya, S. K. (2023). Unveiling the role of gut-brain axis in regulating neurodegenerative diseases: A comprehensive review. *Life Sciences*, 122022.
11. Farshbafnadi, M., Agah, E., & Rezaei, N. (2021). The second brain: The connection between gut microbiota composition and multiple sclerosis. *Journal of Neuroimmunology*, 360, 577700.
12. Altieri, C., Speranza, B., Corbo, M. R., Sinigaglia, M., & Bevilacqua, A. (2023). Gut-microbiota, and multiple sclerosis: background, evidence, and perspectives. *Nutrients*, 15(4), 942.
13. Wang, X., Liang, Z., Wang, S., Ma, D., Zhu, M., & Feng, J. (2022). Role of gut microbiota in multiple sclerosis and potential therapeutic implications. *Current Neuropharmacology*, 20(7), 1413.
14. Zoledziewska, M. (2019). The gut microbiota perspective for interventions in MS. *Autoimmunity Reviews*, 18(8), 814-824.
15. Ullah, H., Tovchiga, O., Daglia, M., & Khan, H. (2021). Modulating gut microbiota: an emerging approach in the prevention and treatment of multiple sclerosis. *Current Neuropharmacology*, 19(11), 1966.
16. Shahi, S. K., Yadav, M., Ghimire, S., & Mangalam, A. K. (2022). Role of the gut microbiome in multiple sclerosis: From etiology to therapeutics. *International review of neurobiology*, 167, 185-215.
17. Kumar, N., Sahoo, N. K., & Mehan, S. (2023). The importance of gut-brain axis and use of probiotics as a treatment strategy for multiple sclerosis. *Multiple sclerosis and related disorders*, 71, 104547.
18. Hashemi, B., Abdollahi, M., Abbaspour-Aghdam, S., Hazrati, A., Malekpour, K., Kafil, H. S., ... & Ahmadi, M. (2023). The effect of probiotics on immune responses and their therapeutic application: a new treatment option for multiple sclerosis. *Biomedicine & Pharmacotherapy*, 159, 114195.
19. Moravejolahkami, A. R., Chitsaz, A., Hassanzadeh, A., & Paknahad, Z. (2023). Effects of anti-inflammatory-antioxidant-rich diet and co-supplemented synbiotics intervention in patients with progressive forms of multiple sclerosis: a single-center, single-blind randomized clinical trial. *Nutritional Neuroscience*, 26(11), 1078-1089.
20. Jiang, J., Chu, C., Wu, C., Wang, C., Zhang, C., Li, T., ... & Chen, W. (2021). Efficacy of probiotics in multiple sclerosis: a systematic review of preclinical trials and meta-analysis of randomized controlled trials. *Food & Function*, 12(6), 2354-2377.

21. Mirashrafi, S., Hejazi Taghanaki, S. Z., Sarlak, F., Moravejolahkami, A. R., Hojjati Kermani, M. A., & Haratian, M. (2021). Effect of probiotics supplementation on disease progression, depression, general health, and anthropometric measurements in relapsing-remitting multiple sclerosis patients: A systematic review and meta-analysis of clinical trials. *International Journal of Clinical Practice*, 75(11), e14724.
22. Rahimlou, M., Hosseini, S. A., Majdinasab, N., Haghighizadeh, M. H., & Husain, D. (2022). Effects of long-term administration of Multi-Strain Probiotic on circulating levels of BDNF, NGF, IL-6 and mental health in patients with multiple sclerosis: a randomized, double-blind, placebo-controlled trial. *Nutritional Neuroscience*, 25(2), 411-422.
23. Dunalska, A., Saramak, K., & Szejko, N. (2023). The role of gut microbiome in the pathogenesis of multiple sclerosis and related disorders. *Cells*, 12(13), 1760.
24. Ma, Y., Sannino, D., Linden, J. R., Haigh, S., Zhao, B., Grigg, J. B., ... & Vartanian, T. (2023). Epsilon toxin-producing Clostridium perfringens colonize the MS gut and epsilon toxin overcomes immune privilege. *J Clin Invest*, 133(9), e163239.
25. Robins, H. F., & Kamarei, A. R. (2021). Beneficial Effect of a Juice-Based Probiotic for Colon Health. *J Gastroenterol Res*, 5(1), 190-196.
26. Robins, H. F., & Kamarei, A. R. (2020). Complete Inhibition of Clostridium difficile with a Probiotic Juice Beverage. *J Gastroenterol Res*, 4(1), 104-111.
27. Wikipedia (2019) Clostridium perfringens. In: Wikipedia. https://en.wikipedia.org/wiki/Clostridium_perfringens
28. CDC (2018) Clostridium perfringens (C. perfringens). In: Centers for Disease Control and Prevention. <https://www.cdc.gov/foodsafety/diseases/clostridium-perfringens.html>
29. McDonel, J. L. (1980). Clostridium perfringens toxins (type a, b, c, d, e). *Pharmacology & therapeutics*, 10(3), 617-655.
30. Songer, J. G. (1996). Clostridial enteric diseases of domestic animals. *Clinical microbiology reviews*, 9(2), 216-234.
31. Johnston MD, Whiteside TE, Allen ME, Kurtz DM (2022) Toxigenic Profile of Clostridium perfringens Strains Isolated from Natural Ingredient Laboratory Animal Diets. *Comparative Medicine* 72:50–58
32. Stiles, B. G., Barth, G., Barth, H., & Popoff, M. R. (2013). Clostridium perfringens epsilon toxin: a malevolent molecule for animals and man?. *Toxins*, 5(11), 2138-2160.
33. Clostridium Perfringens Toxins. In: dph.illinois.gov, <https://dph.illinois.gov/topics-services/emergency-preparedness-response/public-health-care-system-preparedness/clostridium-perfringens-toxins.html>
34. Arizona Department of Health Services (2004) EPSILON TOXIN OF CLOSTRIDIUM PERFRINGENS Bioterrorism Agent Profiles for Health Care Workers.
35. Willis M, Richardson L, Fox S, Sinclair J, Mckee M (2008) Epsilon Toxin from C. perfringens: Characterization of Strain Genotype, Phenotype and Toxin Sensitive Cell Line.

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