UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Biomedicina Comparata e Alimentazione

Corso di Laurea Magistrale in Biotecnologie per l'Alimentazione

# *Antimicrobial activity of bifidobacteria against clostridia species colonizing the digestive tract of infants*

Relatore:
Dott.ssa Maria Elena Martino

Correlatore:
Doc. Ing. Věra Neužil Bunešová, PhD

Laureando: Elena Agostini

Matricola n°: 2022855

ANNO ACCADEMICO 2021/2022

*“Why is a raven like a writing desk?”*

Lewis Carrol, *Alice in Wonderland*

A nonno Elio

# RIASSUNTO

Il microbiota intestinale dei neonati si caratterizza per essere ricco in *Bifidobacterium* spp. I bifidobatteri presentano note capacità di proteggere l'ospite da infezioni enteropatogene attraverso la produzione di acidi grassi a catena corta, come butirrato acetato, e altri meccanismi. D'altra parte, i clostridi sono batteri sporigeni e il genere *Clostridium* comprende specie sia con potenziale probiotico sia altamente patogene.

La presenza e l'attività di batteri sporigeni potenzialmente patogeni nel microbiota intestinale dei neonati è considerata uno dei fattori trigger per l'insorgenza di patologie gastrointestinali, e.g. enterocolite necrotizzante.

La maggior parte degli studi pubblicati si concentra sull'interazione dei bifidobatteri con sole poche specie di clostridi, tra cui *Clostridium perfringens*, *Clostridium butyricum* e *Clostridium difficile*; mentre ricerche su *Clostridium tertium*, *Clostridium neonatale* e altri ancora scarseggiano.

Questo studio si propone di valutare se l'attività antibatterica dei bifidobatteri contro i clostridi patogeni o commensali sia specie o ceppo-specifica e di analizzare come la sensibilità dei clostridi vari tra le specie. Per tanto, lo scopo della tesi consiste nel preparare una panoramica dei bifidobatteri e dei clostridi maggiormente comuni nel tratto digestivo dei neonati e sulla loro interazione, con particolare focus sull'effetto antimicrobico, i.e. sulla capacità dei bifidobatteri di inibire la crescita dei clostridi.

Verranno utilizzati ceppi appartenenti alla collezione del Dipartimento di Microbiologia, Nutrizione e Dietetica della Czech University of Life Sciences Prague (CZU, Praga), ma anche ceppi commerciali provenienti da prodotti probiotici e ceppi appartenenti alla collezione DSMZ (German Collection of Microorganism and Cell Cultures). L'identità di tali ceppi batterici sarà verificata mediante MALDI-TOF MS, mentre i test di attività antimicrobica saranno eseguiti utilizzando il metodo di diffusione, l'agar spot test e l'inibizione della produzione di gas.



**Parole chiave:** attività antimicrobica; bifidobatteri; clostridi; microbiota; probiotici; patogeni

# ABSTRACT

*Bifidobacterium* spp. are dominant taxa of infant microbiota and can protect the host from enteropathogenic infections through acetate production or other mechanisms. Whereas the sporulating clostridia include variable species, from health-promoting butyrate-producing bacteria with the probiotic potential to highly pathogenic bacteria. The presence and activity of potentially dangerous sporulating bacteria in the intestinal microbiota of infants is considered to underlie the etiology of gastrointestinal disorders, such as enterocolitis necrotizing. Most of the published work focuses on the interactions of bifidobacteria with *Clostridium perfringens, C. butyricum* and *C. difficile*, but little is known about *C. tertium, C. neonatale* and others.

We assume that the antimicrobial activity of bifidobacteria against pathogenic or commensal clostridia will be species- or strain-specific. Also, variability between the sensitivity of *Clostridium* spp. will also be found here.
The aim of the thesis will be to prepare an overview about bifidobacteria and clostridia occurring in the digestive tract of infants, including the current findings on their interaction, mainly focused on antimicrobial activity.

Strains from the collection of the Department of Microbiology, Nutrition and Dietetics, Czech University of Life Sciences Prague (CZU, Prague) as well as commercial strains from probiotic products and official type strains obtained from the German Collection of Microorganism and Cell Cultures (DSMZ) will be used for testing. The identity of the strains will be verified using MALDI-TOF MS, while the antimicrobial activity testing will be done using the diffusion method, the agar spot test, and the inhibition of gas production.



**Keywords:** antimicrobial activity; bifidobacteria; clostridia; microbiota; probiotics; pathogen

# TABLE OF ABBREVIATIONS

| | |
|---|---|
| **GIT** | Gastrointestinal tract |
| **SCFAs** | Short-chain fatty acids |
| **NEC** | Necrotizing enterocolitis |
| **AAD** | Antibiotic-associated diarrhea |
| **F6PPK** | Fructose-6-phosphate phosphoketolase |
| **IgA** | Immunoglobulin A |
| **MALDI-TOF MS** | Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry |
| **RT** | Room temperature |
| **NRI** | Not reliably identified |

# INDEX

# 1. INTRODUCTION

## 1.1 THE INTESTINAL MICROBIOTA

Human gastrointestinal tract (GIT), mainly the large intestine, harbours a large number and high diversity of microorganisms. The density of bacterial species has been estimated to exceed $10^{14}$ (Thursby & Juge, 2017). The collection of microbes inhabiting the GIT is known as *gut microbiota* and their collective genome as *microbiome*. Up to 87% of the microbial inhabitants of GIT belonged to two bacterial phyla: *Bacteroides* and *Firmicutes*, which are Gram-negative and Gram-positive bacteria, respectively (Hooper & Macpherson, 2015). The reaming of the intestinal bacterial population (around 10%) belong to *Proteobacteria* and *Actinobacteria*, followed by *Fusobacteria* and *Verrucomicrobia* phyla. At the phylum taxa the pattern of gut microbiota is generally conserved; however, molecular profiling of the human intestinal microbiota revealed a high level of variability at lower-level taxa (genus and species) (Hooper & Macpherson, 2015).

### 1.1.1 Development of gut microbiota

According to di Gioia et al., 2014, the composition of the intestinal microbiota changes during three different stages of life: birth, weaning and the elderly period. Microbial colonization begins soon after the birth and is influenced by several environmental factors such as birth gestational age (full-term or pre-term), delivery method (i.e. vaginal or caesarian) and the type of feeding (i.e. breast milk or formula milk) (Turroni et al., 2018).

Metagenomic and 16S ribosomal RNA (rRNA) gene sequencing studies demonstrated that full-term newborns' microbiota is colonized by a wide variety of microorganisms belonging to *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* genus, whereas the microbiota composition of pre-term infants (< 37 weeks of gestation) is primarily characterized by members of the *Enterobacteriaceae* family (Arboleya et al., 2012, 2015).

According to Walker et al., 2017, the birth process is one of the main factors responsible for shaping the infant gut microbiota, although the colonization may occur prenatally.

Evidence suggest that the vaginally born infants gut microbiota resembles that of the mother's skin and vagina, with the main bacterial taxa being *Enterococcaceae*, *Streptococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Bifidobacteriaceae* (Arrieta et al., 2014). Conversely, infants born through cesarean section are immediately exposed to bacteria that come from the hospital setting and medical personnel. Compared to infants delivered vaginally, their microbiota is characterized by a lower proportion of bifidobacteria and a higher prevalence of clostridia, especially *Clostridium difficile* (Penders et al., 2006). Moreover, literature on infant gut microbiota highlighted that breast-fed infants generally harbour a higher richness and diversity of *Bifidobacterium* spp. than formula-fed infants (Rinninella et al., 2019). Human milk is a rich source of oligosaccharides which are a group of five different monosaccharides (glucose, galactose, N-acetylglucosamine, fucose, and N-acetylneuraminic acid, also known as sialic acid) that are resistant to gastrointestinal digestion. Certain bifidobacteria are responsible for their fermentation to produce short-chain fatty acids (SCFAs), such as acetate, butyrate and propionate, which have a significant impact on human health.

The gut microbiota undergoes another rapid and significant change during weaning, due to the introduction of a variety of novel nutrients. Changes in diet trigger an enrichment of microorganisms belonging to *Clostridium* and *Bacteroides* genus. Interestingly, at this stage the gut microbiota of infants fed either with breast or formula milk become closer to each other (Rinninella et al., 2019).

At approximately three years old, a child's gut microbiota becomes more stable and homogenous. At this phase the composition and diversity are most like those of adults and dominated by three bacterial phyla: *Firmicutes*, *Bacteroides* and *Actinobacteria*. The latter one comprises the bifidobacterial species which are one of the most dominant members of the infant gut microbiota and confer beneficial effects upon their host.

Overall, the gut microbiota has a high degree of variability, and the process of maturation and development is dynamic. Each individual is provided with a unique gut microbiota profile, since this process is influenced by a variety of interrelated factors, including delivery methods, meals, ages, cleanliness, antibiotic use, and genetic factors.

### 1.1.2 Role of gut microbiota in human health

The microbial community that colonized the human GIT establishes a symbiotic relationship with their host, often described as *homeostatic*. The term *homeostasis* refers to any self-regulatory process through which biological systems typically retain stability, while adapting to environmental conditions (Hooper & Macpherson, 2015). The interaction between commensal microorganisms and the host is significant for maintaining the integrity of the mucosal barrier, resulting in the protection against pathogenetic infections. In addition, microorganisms exert important metabolic functions, including the production of vitamins that the host is incapable of producing, and the fermentation of non-digestible fibers, generating metabolites such as SCFAs (Thursby & Juge, 2017). Among the vitamins, gut microbiota can synthesize vitamin K, and B group vitamins, which most commonly synthesized are riboflavin and niacin. Biotin, cobalamin, pantothenic acid, pyridoxine, riboflavin, thiamine, and folate are further notable vitamins (Rowland et al., 2018). On the other hand, the three most common SCFAs are propionate, butyrate, and acetate. They are commonly present in the GIT in a ratio of 1:1:3 and have an impact on intestinal barrier function, epithelium proliferation, and the immune system. Microbial metabolites may affect different cellular processes, including gene expression, chemotaxis, differentiation, proliferation and apoptosis. Moreover, they might modulate the control of appetite and energy intake through receptor-mediated pathways (Thursby & Juge, 2017).

Nevertheless, the mutualistic relationship between gut microbiota and host may be disrupted. A variety of factors, such as changes in diet, use of antibiotics, environmental insults, and immunomodulatory drugs, could alter the microbiota's composition, resulting in both the colonization of potentially pathogenic bacteria and a decline in beneficial species and bacterial diversity (Nagai et al., 2016). An altered microbial composition has been termed *dysbiosis*. Many such studies described associations between dysbiosis and various pathological conditions, including inflammatory bowel disease, irritable bowel syndrome, antibiotic-associated diarrhea (AAD), and necrotizing enterocolitis (NEC). Other evidence reported how microbiota may also be involved in obesity and diabetes (Rowland et al., 2018; Monteiro et al., 2019; Gomaa, 2020).

Therefore, the development of a symbiotic relationship between the host and the microbiota is crucial in order to preserve homeostasis.

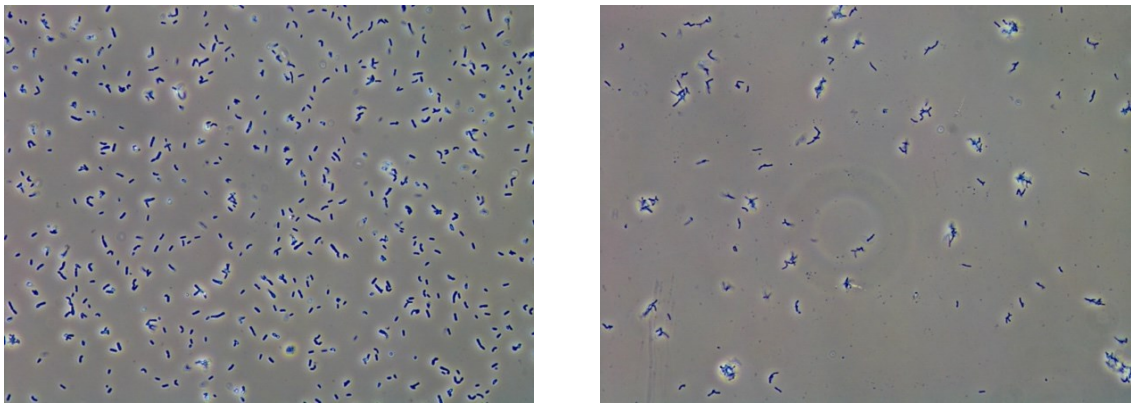
## 1.2 BIFIDOBACTERIA

### 1.2.1 Taxonomy

Bifidobacteria were first isolated from faeces of breast-fed infants by Tissier of the Pasteur Insititute in 1899. Since its bifid shape, it was originally named *Bacillus bifidum*. Consequently, they were classified as members of the genus *Lactobacillus* because of their similarities with lactobacilli. Only in recent times they were recognized as a different genus, named *Bifidobacterium*. Based on the Taxonomic Hierarchy this genus is a member of the *Bifidobacteriaceae* family which belong to the *Actinonobacteria* phylum. Currently, the *Bifidobacterium* genus comprises 109 species with a validly published and correct name, including synonyms (Parte et al., 2020).

### 1.2.2 Morphology

The members of the *Bifidobacterium* genus generally show a bacillus shape with a high level of polymorphism. At the microscope, bifidobacteria generally appear as short and thin rods, with bifurcated or spatulated cellular ends. *Bifidobacterium* spp. can appear as both individual cells and aggregates, although they most frequently form rosettes or a "V" or palisade of parallel cells (Fig. 1). The colonies that *Bifidobacterium* spp. produces are soft, convex, cream- or white-coloured, shiny, and smooth.



(a) (b)

**Figure 1.** (a) Individual cells of *B. animalis* BEBA Nestlè; (b) Auto-aggregation of *B. bifidum* NORF 78/9 cells.

### 1.2.3 Physiology

*Bifidobacterium* spp. are Gram-positive microorganism whit a high G+C DNA content. They basically stand out by being no-spore forming and catalase-negative bacteria. Moreover, they grow in the range temperature of 25 - 45°C and in anaerobic conditions, although some members of the *Bifidobacterium* genus may grow aerobically with an enriched atmosphere containing 10 % $CO_2$ (Andrade et al., 2020). Bifidobacterial species are characterized by carbohydrate metabolism which results in the production of metabolites, including acetic acid, lactic acid, formic acid, succinic acid, and ethanol. Especially they are able to utilize different carbon sources that escape degradation in the upper part of GIT, such as gastric mucin, malto-oligosaccharides, fructo-oligosaccharides, pectin and other plant derived-oligosaccharides (Pokusaeva et al., 2011).

The pathway of carbohydrate metabolism occurring in the *Bifidobacterium* genus is named *bifidus shut* or *fructose-6-phoshate shunt*, as fructose-6-phosphate phosphoketolase (F6PPK) is the key enzyme. In addition, the presence of F6PPK activity is considered a taxonomic marker for bifidobacterial species (Modesto et al., 2021).

### 1.2.4 Importance of bifidobacteria in infant's intestinal homeostasis

Bifidobacteria are among the most prevalent inhabitants of infant gut microbiota. They are abundant in the large intestine, especially in the proximal colon. The members of

*Bifidobacterium* genus that usually colonize the human microbiota are 9, represented by *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. dentium*, *B. catenulatum*, *B. pseudocatenulatum, B. longum*, and *B. pseudolongum*. Evidence of recent studies demonstrated that the presence of different species of bifidobacteria changes with age, from childhood to old age. Interestingly, adults frequently have higher populations of the species *B. adolescentis*, *B. pseudocatenulatum*, and *B. catenulatum*, whereas *B. breve*, *B. bifidum*, *B. longum* subsp. *longum*, and *B. longum* subsp. *infantis* are the most prevalent species in infants (Saturio et al., 2021; Turroni et al., 2018). However, the difference between the bifidobacterial species of a newborn and an adult is not pretty tight, since some strains were transferred from mother to infant. Milani et al., 2015 provided evidence of vertical transmission of bifidobacterial species, revealing the existence of shared bifidobacterial strains (*B. breve* and *B. longum* subsp. *longum*).

The physiology and pathophysiology of humans are positively impacted by a variety of *Bifidobacterium* spp.. Their ability to protect the host is related to bacterial fermentation that results in short-chain fatty acid production, such as acetic acid, butyric acid, and propionic acid. They promote gut homeostasis through different mechanisms, including enhancement of mucus by intestinal cells, activation of inflammasomes, and increased secretion of immunoglobulin A (IgA) (Rooks & Garrett, 2016). Among the SCFAs, butyrate is crucial for preserving health, since it regulates the immune system and maintains epithelial barrier function (Baxter et al., 2019). *Bifidobacterium longum* subsp. *longum* may protect from enteropathogenic infection through the production of acetate, as demonstrated by Fukuda et al., 2011. Additionally, according to Thursby & Juge (2017), bifidobacteria are the primary producers of folate, a vitamin essential for essential host metabolic processes including DNA synthesis and repair.

Overall the presence and abundance of bifidobacteria in the human gut are related to health status, since they protect against early-life diseases including necrotizing enterocolitis, diarrhea, ulcerative colitis and constipation. This makes them potential microbial biomarkers (Milani et al., 2017).

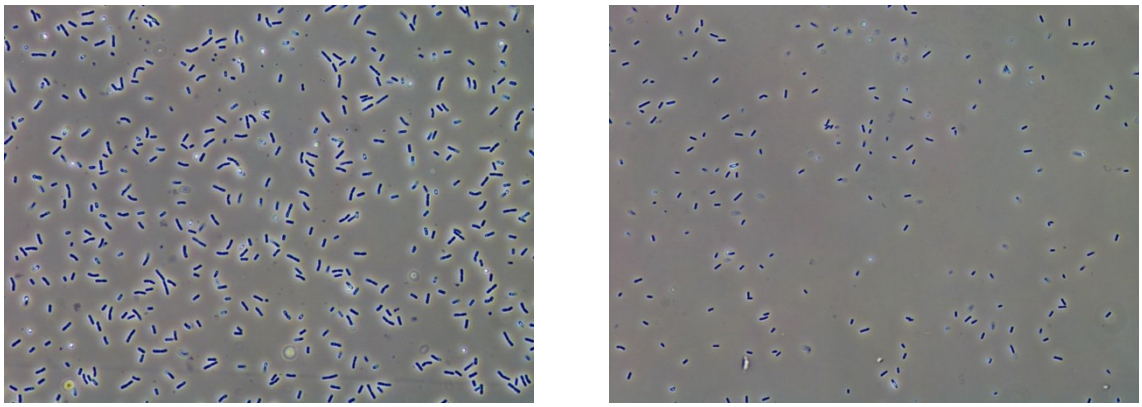
Conversely, some diseases are linked to reduced levels of bifidobacteria in the human GIT. In addition, the use of antibiotics causes a further decline in *Bifidobacterium* populations in favour of an increased abundance of *Proteobacteria*.

This suggests the important role of *Bifidobacterium* spp. in establishing intestinal homeostasis and the potential use as biomarkers to evaluate the intestinal condition in relation to a potential dysbiosis.

## 1.3 CLOSTRIDIA

### 1.3.1 Genetic characteristics of clostridia

The *Clostridium* genus is one of the largest bacteria genera belonging to the phylum of *Firmicutes* and the family *Clostridiaceae*. Rod-shaped structures are distinctive to members of this genus and can appear singly, in pairs, or in short chains (Fig. 2).



**(a)** **(b)**

**Figure 2.** (a) *C. butyricum* CP_NI11 arranged singly; (b) *C. perfringens* CP_NI 17 arranged in pairs.

*Clostridium* spp. are Gram-positive bacteria and the majority of species are obligate anaerobes. They are catalase-negative and fermentative. Members of the *Clostridium* genus are particularly adaptable in their metabolic processes and can degrade a variety of organic substances, including carbohydrates, organic acids, alcohols, aromatic compounds, peptides, aminoacids, amines, purines, and pyrimidines (Popoff & Bouvet, 2013). Clostridia have the ability to produce endospores which ensure their survival under adverse conditions for long periods. The germination of spores is often induced by various amino acids, often in combination with phosphate and sodium (Shen et al., 2019).

### 1.3.2 Commensal and pathogen clostridia

*Clostridium* genus is vast and heterogeneous, since it includes both commensal bacteria, which are involved in the maintenance of gut homeostasis, and pathogenic members, which may cause several gastrointestinal disorders. The former ones interact with other microorganism in GIT modulating physiologic, metabolic and immune processes. Conversely, evidence suggests that the pathogenic species exhibit cytotoxic activity, mostly resulting in pathogenesis of NEC (Schönherr-Hellec et al., 2018). NEC is the most common and serious gastrointestinal disorder among newborn infants and the clinical symptoms include abdominal distension, gastrointestinal bleeding, mucosal ulcerations and necrosis, portal venous gas, and pneumatosis intestinalis, with different degrees of severity (Cassir et al., 2016). Genetic susceptibility, intestinal immaturity, and alterations in microvascular tone are risk factors for this illness; nevertheless, clostridia-colonized newborns exhibit a faster illness progression (Meister et al., 2020; Schönherr-Hellec & Aires, 2019).

Genetic studies have been mainly focused on *Clostridium perfringes* and *Clostridium difficile*. According to Uzal et al., 2014, *Clostridium perfringens* is able to produce toxins (CPA and PFO) which interfere with the immune response, resulting in host cell leakage and lysis. Due to this, the pathogen is considered responsible for approximately 5 – 15% of all cases of antibiotic-associated diarrhea (AAD), which develops in 5 – 40% of all patients receiving antibiotic therapy. Similarly, *C. difficile* causes gastrointestinal disorders through the action of two toxins: toxin A (TcdA) and toxin B (TcdB). The majority of *C. difficile* infections are nosocomial and are responsible for 30% of AAD, with clinical symptoms varying from mild diarrhea to severe complications associated with pseudomembranous colitis, toxic megacolon and death (Sorg et al., 2019).

Together with *C. perfringens* and *C. difficile*, evidence points to a connection between *C. butyricum* and NEC (Schönherr-Hellec et al., 2018). Even though this pathogen is classified as commensal bacteria, recent research reported that some *C. butyricum* strains express virulence factors, such as enterotoxins and neuraminidase (Cassir et al., 2016; Ariyoshi et al., 2022).

## 1.4 Background research on the interaction between bifidobacteria and clostridia

Many researchers have investigated the ability of bacteria to produce antimicrobial compounds against pathogens. The *antimicrobial activity* term refers to the production of antibacterial substances which includes organic acids, hydrogen peroxide and bacteriocins. By producing SCFAs and lowering luminal pH, these substances primarily contribute to the decrease of pathogens' viability as well as metabolisms and toxin generation (Adak et al., 2019). The *in vitro* antimicrobial activity might be evaluated with several methods and the most well-known and standard techniques are the disk-diffusion and broth or agar dilution procedures (Balouiri et al., 2016). Other processes that can contribute to antimicrobial sensitivity include the augmentation of the gut epithelium, colonization competition, and/or stimulation of the innate immune response (Golić et al., 2017).

Previously published studies on bacteria's antimicrobial activity are limited to a few interactions between bifidobacteria and clostridia. Among these studies, Yun et al., 2017 reported that *B. longum* produced acid organic, in particular lactic acid, that led to the lowering of pH and consequently the inhibition of *C. difficile*, when cocultured. The physiological activities of this pathogenic bacteria may also be suppressed by *B. breve.* This bifidobacteria has the ability to prevent the generation of spores, biofilm, toxins, and virulence genes (Sorg et al., 2019;Yang & Yang, 2019). Moreover, *B. breve* may damage the permeability and integrity of *C. difficile* cell membrane, allowing intercellular substances to flow out (Rui et al., 2022).

## 1.5 Bifidobacteria as potential probiotic

Probiotics are defined as "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" according to the International Scientific Association for Probiotics and Prebiotics. Several criteria must be fulfilled in order to qualify microorganisms as probiotics. Probiotic strains must be (i) *sufficiently characterized*, (ii) *safe for the intended use,* (iii) *supported by at least one successful*

*human clinical trial carried out in accordance with generally accepted scientific standards,* and (iv) *alive in sufficient numbers in the product at an effective dose throughout shelf life* (Binetti et al., 2020). In addition to these requirements, microorganisms might have the ability to coaggregate with microbial pathogens, adhere to eukaryotic cells and mucus, and tolerate conditions of acidic pH and bile salts (Golić et al., 2017). The latter condition varies depending on taxa, species, and strains: bifidobacteria are generally less acid-tolerant than lactobacillus but may withstand high bile salt concentrations (Santos do Carmo et al., 2018).

This genus has been extensively studied in recent years, due to its important role in the human gut microbiota and the widespread use of certain bifidobacterial strains as probiotic products to prevent and guard against dysbiosis in early life.

Among *Bifidobacterium* spp, evidence suggests how *B. breve* can promote bifidobacterial colonization and protect preterm infants from NEC by producing SCFAs that may have an impact on the integrity and health of the intestinal epithelium and immune cells (Wong et al., 2019).

In addition, the recent study by Cukrowska et al., 2020 indicated that this strain has the capacity to increase secretory IgA synthesis, preventing the development of allergies. Along with *B. breve*, *B. infantis* contributes to the reduction of allergic inflammation (Liu et al., 2017). Another example is provided by *B. animalis* subsp. *lactis* BB12, which prevents from a reduction in faecal acetate levels in subjects receiving antibiotics, according to Merenstein et al., 2021.

Overall among the several strains, studies reported how *Bifidobacterium* spp. might be used as probiotics for therapeutic purposes in infants, since their administration may result in the prevention of NEC and reduction in the risk as well as treatment of infectious and atopic illness.

The purpose of this study is to investigate if bifidobacteria have antibacterial activity against different clostridial species, with a focus on whether this activity is strain- or species-specific.

# 2. MATERIALS AND METHODS

## 2.1 Bacterial strains and growth condition

In this study a total a of 29 bifidobacterial and 17 clostridial strains were investigated.

### 2.1.1 Bifidobacterial strains

The tested bifidobacterial strains tested belonged to the following species: *Bifidobacterium adolescentis* (= 3), *Bifidobacterium animalis* (*n* = 9), *Bifidobacterium bifidum* (*n* = 4), *Bifidobacterium breve* (*n* = 7), *Bifidobacterium catenulatum* (*n* = 2), *Bifidobacterium longum* (*n* = 1), *Bifidobacterium pseudocatenulatum* (*n* = 1), and *Bifidobacterium pseudolongum* (*n* = 2). For each bifidobacteria two or more strains were investigated. *Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum* are two exceptions, both of which only had one strain examined, named *Bifidobacterium longum* NORF 79/8A, and *Bifidobacterium pseudocatenulatum* MOTOL 1/8A.

The majority of the strains tested were isolated from faecal samples of newborn infants, up to 2 years old. Some strains represented by *Bifidobacterium adolescentis* MB 10/1, *Bifidobacterium adolescentis* MŠ B2, *Bifidobacterium adolescentis* MŠ B3, *Bifidobacterium animalis* BN and *Bifidobacterium pseudocatenulatum* MOTOL 1/8A were isolated from faecal samples of adults. In this study were also included three strains (*Bifidobacterium animalis* BB12, *Bifidobacterium bifidum* (TMT) NUTRA BONA, and *Bifidobacterium breve* BR03 probiotics drops) which were obtains from probiotic products and two strains (*Bifidobacterium animalis* subsp. *lactis* DSM 10140 and *Bifidobacterium animalis* DANONE) isolated from yogurt. Moreover, the strain *Bifidobacterium animalis* Nestlé was isolated from infant nutrition (Tab. 1).

Overall, a total of 29 bifidobacteria were investigated in this study, with *Streptococcus thermophilus* used as a positive control.

The isolates were routinely cultured into glass tubes containing 9 ml of sterile Wilkins-Chalgren broth (33 g/L, Oxoid, UK) supplemented with GMO-Free soya peptone (5 g/L, Oxoid, UK), L-cysteine (0.5 g/L, Sigma-Aldrich, USA), and Tween 80 (Polysorbate 80) (1 mL/L, Sigma-Aldrich, USA). The WSP broth was prepared according to the rool tube technique (Hungate method) that allow to achieve an oxygen-free carbon dioxide

environment (Attebery & Finegold, 1969). Bifidobacteria were incubated at 37°C for 24 h.

### 2.1.2 Clostridial strains

The following clostridia strains were studied: *Clostridium butyricum* A74, *Clostridium butyricum* 10702*, Clostridium butyricum* CP_NI 11*, Clostridium butyricum* CP_NI 18*, Clostridium perfringens* C68, *Clostridium perfringens* 11778*, Clostridium perfringens* CP_NI 14, *Clostridium perfringens* CP_NI 17*, Clostridium difficile* A28, *Clostridium difficile* 3593, *Clostridium difficile* 12056, Clostridium *tertium* A33*,* Clostridium *tertium* CP_NI 27, *Clostridium neonatale* L1, *Clostridium paraputrificum* C91, *Clostridium paraputrificum* 2630, and *Clostridium clostridioforme* 933 *.*

Among the 17 clostridial isolates studied, 11 (*Clostridium butyricum* A64, *Clostridium butyricum* CP_NI 11, *Clostridium butyricum* CP_NI 18, Clostridium perfringens C68, *Clostridium perfringens* CP_NI 14, *Clostridium perfringens* CP_NI 17, Clostridium *difficile* A28, *Clostridium tertium* A33, *Clostridium tertium* CP_NI 27, *Clostridium neonatale* L1, and *Clostridium paraputrificum* C91) were isolated from faecal samples of newborn infants at 6 months old age and 6 (*Clostridium butyricum* 10702, *Clostridium perfringens* 11778, *Clostridium difficile* 3593, *Clostridium difficile* 12056, *Clostridium paraputrificum* 2630, and *Clostridium clostridioforme* 933) were obtained from the German Collection of Microorganism and Cell Cultures (DSMZ, Braunschweig-Süd, Germany).

Overall a total of 17 *Clostridium* strains were investigated in this study (Tab. 2).

Same as bifidobacterial strains, clostridial isolates were routinely cultures into glass tubes filled with WSP broth at 37°C for 24-48 h.

## 2.2 MALDI-TOF MS-based identification of bacterial cultures

All the strains investigated in this study were identified using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Microorganisms are identified by creating mass spectra from whole cells or isolated intracellular content, which are then compared to known database references (Vaishampayan et al., 2018). For this procedure, 1 ml of each fresh culture was transferred into apposite Eppendorf tubes. The cell suspensions were centrifugated at

14500 rpm for 2 minutes and then the supernatants were discarded. Using a pipette, the pellets were resuspended in 500 µl of 70% ethanol, and the samples were centrifugated once more at 14500 rpm for 2 minutes. Supernatants were discarded and the residual drops at the bottom of each tube were extracted with a pipette. The pellets were left to air dry at room temperature (25-28°C, RT) for at least 10 minutes, in order to let the residual ethanol evaporate. Then, everything was resuspended with 15 µl of 70% formic acid (formic acid was stored at 4-7°C) followed by an equal volume of acetonitrile (acetonitrile was stored at RT). Subsequently, the samples were vortexed following a centrifugation step at 14500 rpm for 2 minutes. Then, 1 µl of the supernatant was inoculated on the relevant position of a MALDI target place and allowed them to air dry at RT. As final step, the sample spots were overlaid with 1 µl of a matrix solution containing α-Cyano-4-hydroxycinnamic acid (α-CHCA, Sigma-Aldrich, USA) and let them air dry at RT.

The following scores were assigned to each identification according to the Bruker criteria. The level of similarity between an unknown sample and a reference one was expressed by a log(score): > 2.30 was regarded as *highly probable species identification;* 2.00 - 2.30 as *secure genus identification and probable species identification; 1.70 – 1.99* as *probable genus identification;* and < 1.70 interpreted as *not reliably identified* (NRI). Only the closest type strain match (the highest score value) was recorder as potential species identification (Normand et al., 2017; Vaishampayan et al., 2018).

## 2.3 Freezing of bacterial cultures

The stock culture of both bifidobacterial and clostridia isolates was stored in the Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, at the Czech University of Life Science CZU, Prague, Czech Republic. An aliquot of 1.2 ml of each fresh bacterial culture was put into a 2.0 ml cryotube containing 0.7 ml of BifiBuffer (1.2 g/L K2HPO4, 0.333 g/L KH2PO4 and 0.5 g/L cystein) supplemented with glycerol.

The bacterial cultures were also stored either in glass penicillins at -20 °C containing 9 ml of WSP broth and 6 ml of BifiBuffer + glycerol or in cooked meat medium (CMM).

## 2.4 Preparation of fresh bacterial culture and purity check

Bifidobacterial and clostridial strains were injected into 9 ml of WSP broth (anaerobic environment) in 0.3 ml portion, and they were incubated for 24 h at 37°C. After 24 h of incubation, the freshly grown cultures were evaluated for their purity through optical microscopy. The microscope slides were prepared by depositing one drop of each sample and covering it with a slip. A 40x objective lens was used to view the samples using the Nikon Eclipse E200 phase-contrast microscope. The cultures which appeared to be pure were used for next testing, whereas the contaminated ones were discarded.

### 2.5 Antimicrobial activity screening

The antimicrobial activity evaluation of *Bifidobacteria* spp. against clostridia was carried out according to three methods: agar well diffusion method, agar spot test and inhibition of gas production.

#### 2.5.1 Agar well diffusion method

The production of antimicrobial substances by bifidobacteria was assessed according to the agar well diffusion method described by Tagg & Mcgiven, 1971, with some modifications. In this analysis an aliquot of 1 ml of each overnight culture of *Clostridium* spp. was inoculated into the Petri dishes (Ø 90 mm) with 20 ml of Anaerobe Basal Agar (Oxoid, UK). The culture medium was kept at RT in anaerobic conditions until complete solification. Then, 6 mm diameters wells were created leaving a spot for the following step. An amount of 50 µl of each bifidobacterial supernatants was used to fill the wells. The plates were incubated under anaerobic conditions at 37°C for 48h in order to allow colonies to develop. The bacterial lawns were examined for zones of inhibition surrounding the wells filled with bifidobacterial supernatants. The absence of clostridial growth, as revealed by the existence of a clean zone surrounding the holes (> 7 mm), indicates the presence of antimicrobial activity. The diameters of inhibitory halos were eventually measured and expressed in millimeters. Based on the breadth of the inhibition zone, the bifidobacterial strains were classified as having low (7 - 8 mm),

medium (9 – 13 mm) or high (> 13 mm) ability to inhibit the growth of clostridial species (Fig. 3).

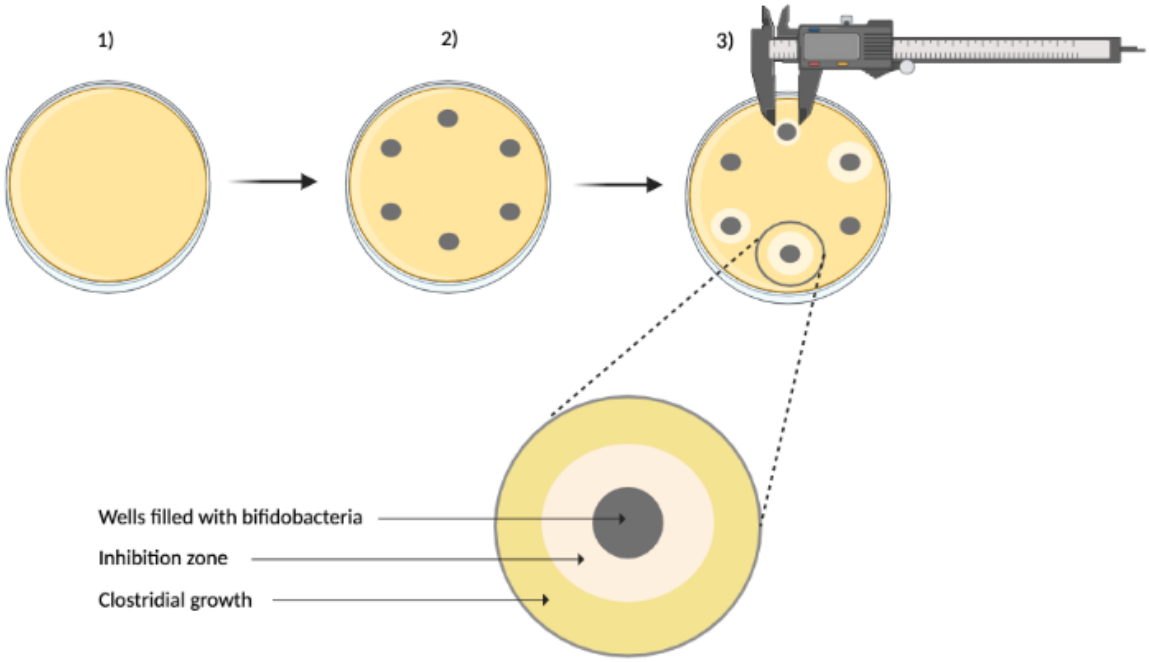



**Figure 3.** Agar well diffusion method. 1) Inoculation of clostridial culture on Anaerobe Basal Agar plate 2) Addition of bifidobacterial supernatant into the wells 3) Measurement of the inhibition zone.

### 2.5.2 Agar spot test

The ability of bifidobacteria to inhibit the growth of clostridia species was also evaluated by assessing the formation of a clear halo around their growth. The agar spot test was performed as described by Monteiro et al., 2019, with some modifications. The Petri dishes (Ø 90 mm) were filled with 10 ml of WSP Agar and the culture media was solidified at RT for 24 h. After solidification of the culture medium, 1 µl (~ $10^7$ CFU) of each potential probiotic culture was spotted onto one half-circle of the culture medium and the plates were incubated at 37°C for 24 h under anaerobic conditions. After 24 h, 40 µl *Clostridium* spp. cultures were homogenized with 10 ml of Anaerobe Basal Agar and overlaid onto the WSP Agar containing already the grown bifidobacteria. Everything was cultivated at 37°C for 24 h in anaerobic conditions. A positive essay for antimicrobial activity was characterized by the formation of a clear zone around the growth of the

probiotics, which was measured and expressed in millimeters. The bifidobacterial strains were classified as having low (7 - 10 mm), medium (11 - 15 mm), or high (> 15 mm) antimicrobial activity based on the width of the inhibitory zone (Fig. 4).

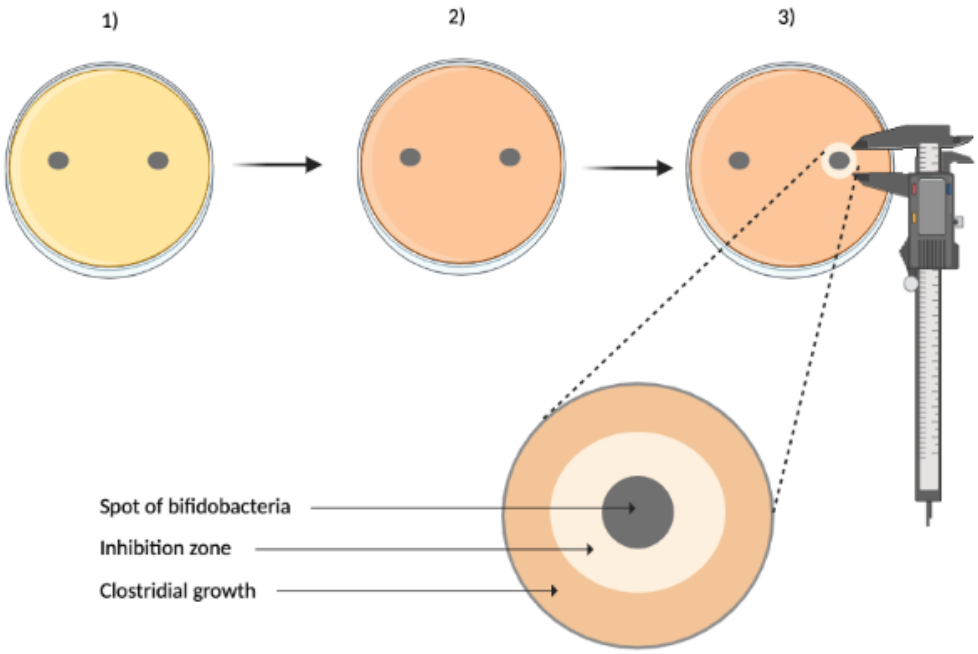



**Figure 4.** Agar spot test. 1) Spotting of bifidobacterial strains onto one half-circle of the WSP Agar plate 2) Addition of clostridial cultures homogenized with Anaerobe Basal medium 3) Measurement of the inhibition zone.

### 2.5.3 Inhibition of gas production

The ability of bifidobacteria to inhibit the gas production of clostridial species was evaluated according to the method described by Golić et al., 2017, with some modifications. This essay was performed by filling each of the 15 ml capacity sterile plastic tube with 3 ml of the Clostridium difficile Agar (Oxoid, UK) and then inoculating 600 µl of each overnight culture of *Clostridium* spp. into the upper third layer. After

solidification, the culture medium was overlaid with 3 ml of MRS Agar (Oxoid, UK) followed by 120 µl of bifidobacterial cultures and incubated under anaerobic conditions at 37°C for 24 h. The growth of clostridial species was characterized by the turbidity of the medium and the gas production by the presence of bubbles in the culture media (Monteiro et al., 2019). The reduction and the absence of bubbles in the culture media were classified as positive and partial positive essay, respectively (Fig. 5).


**Figure 5.** Inhibition of gas production. 1) Inoculation of clostridial strains into tube following by Clostridium difficile Agar 2) Addition of MRS Agar and bifidobacterial culture 3) Evaluation of the presence of bubbles in the culture media.

# 3. RESULTS

## 3.1 MALDI-TOF MS-based identification of bacterial isolates

MALDI-TOF MS was performed in order to re-identify all the strains tested in this study.

### 3.1.1 Bifidobacterial isolates

The MALDI BioTyper-based identification of the *Bifidobacterium* spp. classified the majority of the samples (89.65%) as belonging to the genus *Bifidobacterium*. In Table 1 is shown how of to the 29 samples analyzed, 4 (*Bifidobacterium adolescentis* MŠ B3, *Bifidobacterium animalis* BN, *Bifidobacterium catenulatum* DSM 16992, *Bifidobacterium longum* B28, and *Bifidobacterium pseudolongum* E43) reported the highest log(score) values (> 2.300), thus they were identified at the species taxa with a *high probability* (green range). The green range with the score between 2.00 and 2.30 also includes all the samples classified as *surely genus identified and probably species identified,* which were 18. Among all the samples identified, 4 represented by *Bifidobacterium animalis* Nestlé, *Bifidobacterium bifidum* (TMT) NUTRA BONA, *Bifidobacterium breve* NORF 78/9, and *Bifidobacterium bifidum* E13y got a log(score) between 1.70 and 2.00, which means that the identification at the genus taxa is possible (yellow range). Only 3 isolated, represented by *Bifidobacterium bifidum* NORF 78/9, *Bifidobacterium breve* J41, and *Bifidobacterium catenulatum* D16, were classified as *not reliably identified* (red range).
When comparing the results of MALDI-TOF to the previous ones, the identity of 6 samples at the species level turned out not to be correct: the type strains originally labeled as *Bifidobacterium bifidum* NORF 78/9 B, *Bifidobacterium catenulatum* B46, *Bifidobacterium longum* BLON, *Bifidobacterium longum* E13y, *Bifidobacterium longum* D15 and *Bifidobacterium longum* B28 were identified respectively as *Bifidobacterium breve*, *Bifidobacterium animalis*, *Bifidobacterium pseudolongum*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, and *Bifidobacterium animalis*.

Among the bifidobacterial strains identified using Bruker Daltonics criteria, 22 (75,86 %) were classified at the log-score values > 2.00 (green range), 4 (13.80 %) between 1.70 and 2.00 (yellow range) and 3 (10.34 %) < 1.70 (red range).

**Table 1**. Type and collection strains of *Bifidobacterium* genus.

| ***Bifidobacterium*** **species** | **Strains code** | **Origin** | **MALDI-TOF identification** | **Score** |
|---|---|---|---|---|
| *Bifidobacterium adolescentis* | MB 10/1 | Human (adult) | *Bifidobacterium adolescentis* | 2.07 |
| *Bifidobacterium adolescentis* | MŠ B2 | Human (adult) | *Bifidobacterium adolescentis* | 2.11 |
| *Bifidobacterium adolescentis* | MŠ B3 | Human (adult) | *Bifidobacterium adolescentis* | 2.34 |
| *Bifidobacterium animalis* | BB12 | Probiotic product | *Bifidobacterium animalis* | 2.17 |
| *Bifidobacterium animalis* | BN | Human (adult) | *Bifidobacterium animalis* | 2.37. |
| *Bifidobacterium animalis subsp. lactis* | DSM 10140 | Yogurt | *Bifidobacterium animalis* | 2.16 |
| *Bifidobacterium animalis* | DANONE | Yogurt | *Bifidobacterium animalis* | 2.14 |
| *Bifidobacterium animalis* | Neslè | Infant nutrition | *Bifidobacterium animalis* | 1.16 |
| *Bifidobacterium animalis* | MUP 74/7b | Human (infant) | *Bifidobacterium animalis* | 2.26 |
| *Bifidobacterium bifidum* | MA1 | Human (infant) | *Bifidobacterium bifidum* | 2.01 |
| *Bifidobacterium bifidum* | (TMT) NUTRA BONA | Probiotic product | *Bifidobacterium bifidum* | 1.7 |
| *Bifidobacterium bifidum* | NORF 78/9 B | Human (infant) | *Bifidobacterium breve* | 1.92 |
| *Bifidobacterium bifidum* | NORF 78/9 | Human (infant) | NRI | 1.6 |
| *Bifidobacterium breve* | BR03 probiotics drops | Probiotic product | *Bifidobacterium breve* | 2.18 |
| *Bifidobacterium breve* | MUP 78/7B | Human (infant) | *Bifidobacterium breve* | 2.12 |
| *Bifidobacterium breve* | J19 | Human (infant) | *Bifidobacterium breve* | 2.11 |
| *Bifidobacterium breve* | J41 | Human (infant) | NRI | 1.53 |
| *Bifidobacterium breve* | B42 | Human (infant) | *Bifidobacterium breve* | 2.05 |
| *Bifidobacterium breve* | MUP 77/7a | Human (infant) | *Bifidobacterium breve* | 2.11 |
| *Bifidobacterium catenulatum subps. catenulatum* | DSM 16992 | human (adult) | *Bifidobacterium catenulatum* | 2.33 |
| *Bifidobacterium catenulatum* | D16 | Human (infant) | NRI | 1.52 |
| *Bifidobacterium catenulatum* | B46 | Human (infant) | *Bifidobacterium animalis* | 2.02 |
| *Bifidobacterium pseudocatenulatum* | MOTOL 1/8A | Human (adult) | *Bifidobacterium pseudocatenulatum* | 2.28 |
| *Bifidobacterium longum* | BLON | Human (infant) | *Bifidobacterium pseudolongum* | 2.2 |
| *Bifidobacterium longum* | E13y | Human (infant) | *Bifidobacterium bifidum* | 1.7 |
| *Bifidobacterium longum* | NORF 79/8A | Human (infant) | *Bifidobacterium longum* | 2.11 |
| *Bifidobacterium longum* | D15 | Human (infant) | *Bifidobacterium animalis* | 2.12 |
| *Bifidobacterium longum* | B28 | Human (infant) | *Bifidobacterium animalis* | 2.31 |
| *Bifidobacterium pseudolongum* E43 | E43 | Human (infant) | *Bifidobacterium pseudolongum* | 2.38 |

Reliable species identification

Reliable genus identification

No reliable identification

### 3.2.2 Clostridial isolates

Almost all of the strains (94,12 %) were correctly re-identified as belonging to *Clostridium* genus. The only exception was *Clostridium neonatale* L1 which was interpreted as *not reliably identified* (NRI, red range), since the database didn't contain his representative peptide mass fingerprint. According to MALDI-TOF results (Tab. 2) the strains *Clostridium tertium* A33, *Clostridium tertium* CP_NI 27, and *Clostridium clostridioforme* 933 reported the best identification matches (log(score) > 2.300), which referred to a *high probable identification at the species level* (green range).

Four strains, *Clostridium perfringens* CP_NI 14, *Clostridium difficile* A28, *Clostridium difficile* DSM 1296, and *Clostridium paraputrificum* DSM 2630 were classified as *probably identified at the genus level* (yellow range) as they got a log(score) value between 1.70 and 1.99.

Overall 12 clostridial species (70.58 %) were classified at the log-score values > 2.00 (green range), 4 (23.53 %) between 1.70 and 2.00 (yellow range) and 1 (5.89 %) < 1.70 (red range).

**Table 2.** Type and collection strains of *Clostridium* genus.

| ***Clostridium*** **species** | **Strain code** | **Origin** | **MALDI-TOF identification** | **Score** |
|---|---|---|---|---|
| *Clostridium butyricum* | A74 | Human (infant) | *Clostridium butyricum* | 2.21 |
| *Clostridium butyricum* | DSM 10702 | Intestine of pig | *Clostridium butyricum* | 2.18 |
| *Clostridium butyricum* | CP_NI 11 | Human (infant) | *Clostridium butyricum* | 2.02 |
| *Clostridium butyricum* | CP_NI 18 | Human (infant) | *Clostridium butyricum* | 2.09 |
| *Clostridium perfringens* | C68 | Human (infant) | *Clostridium perfringens* | 2.34 |
| *Clostridium perfringens* | DSM 11778 | Boulette (Hamburger) | *Clostridium perfringens* | 2.11 |
| *Clostridium perfringens* | CP_NI 14 | Human (infant) | *Clostridium perfringens* | 1.96 |
| *Clostridium perfringens* | CP_NI 17 | Human (infant) | *Clostridium perfringens* | 2.2 |
| *Clostridium difficile* | A28 | Human (infant) | *Clostridium difficile* | 1.72 |
| *Clostrium difficile* (syn. *Clostridioides difficile*)[1] | DSM 1296 | HUman (adult) | *Clostridium difficile* | 1.88 |
| *Clostrium difficile* (syn. *Clostridioides difficile*)[1] | DSM 12056 | Rumen of new-born lamb | *Clostridium difficile* | 2.06 |
| *Clostridium tertium* | A33 | Human (infant) | *Clostridium tertium* | 2.31 |
| *Clostridium tertium* | CP_NI 27 | Human (infant) | *Clostridium tertium* | 2.38 |
| *Clostridium neonatale* | L1 | Human (infant) | NRI | 1.58 |
| *Clostridium paraputrificum* | C91 | Human (infant) | *Clostridium paraputrificum* | 2.11 |
| *Clostridium paraputrificum* | DSM 2630 | Unknown | *Clostridium paraputrificum* | 1.88 |
| *Clostridium clostridioforme* (syn. *Enterocloster clostridioformis*)[2] | DSM 933 | Calf rumen | *Clostridium clostridioforme* | 2.34 |

[1] Reclassification according to Lawson et al., 2016.

[2] Reclassification according to Haas & Blanchard, 2020.

Reliable species identification

Reliable genus identification

No reliable identification

## 3.2 Antimicrobial activity evaluation

A total of 29 bifidobacterial strains were used in this analysis. The highest percentage of bifidobacteria were isolated from human faecal samples, followed by probiotic products and yogurt. All of the strains were tested against 7 different clostridial species, for a total of 17 strains collected either from human or animals' samples. In this study, the ability of bifidobacteria to inhibit clostridial growth was assessed through three different methods, which are the agar spot test, the diffusion method, and the inhibition of gas

production. Among all of them, the most successful results were achieved with the former one. Describing the antimicrobial activity in the agar spot test and in the diffusion method, the score of 6 is relating to a negative result, as it represents the diameters both of the spot and wells. Additionally, the *no detected* caption has been provided in the agar spot and gas production data tables. This is due to the laboratory complication associated with clostridia's growth problem.

### 3.2.1 Diffusion method

The antimicrobial activity of *Bifidobacterium* spp. was evaluated with the diffusion method after 24 h of incubation. The results are shown in the table below (Tab. 3) where the potential probiotics were classified based on the inhibition of halos' diameters. They are grouped together between 7 mm and 8 mm, between 9 mm and 13 mm, and over 13 mm ranges.

The species that produced the best outcomes are represented by *Bifidobacterium breve* NORF 78/9 and *Bifidobacterium pseudocatenulatum* MOTOL 1/8A, which reached the score of 18 mm against *C. butyricum* 10702*,* followed by *B. bifidum* (TMT) NUTRA BONA and *B. breve* J19, which got a score of 15 and 14 mm against *C. butyricum* A74.

What is interesting about the data in this table is that the majority of *Bifidobacterium breve* strains, isolated from infant fecal samples, had inhibitory effects on both *C. difficile* and *C. neonatale* growth. Due to their ability to suppress both pathogens, 4 to 7 strains of *B. breve* (MUP 78/7B, J19, B42, MUP 77/7a) exhibited a good antimicrobial action, with halos ranging from 8 to 11 mm. The data also shows that *Bifidobacterium animalis* strains had a modest antibacterial impact on the majority of clostridial strains, despite the fact that they did not produce significant halos.

Additionally, no significant results were found among *B. adolescentis*, *B. longum* and *B. pseudolongum*.

**Table 3.** Antimicrobial susceptibility of *Bifidobacterium* spp. against *Clostridium* spp. by the diffusion method.

Diameter of Inhibition Zones (mm)

| Potential Probiotics | *Clostridium butyricum* | | | | *Clostridium perfringens* | | | | *Clostridium difficile* | | | *Clostridium tertium* | | *Clostridium neonatale* | *Clostridium paraputrificum* | | *Clostridium clostridioforme* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A74 | 10702 | CP_NI 11 | CP_NI 18 | C68 | 11778 | CP_NI 14 | CP_NI 17 | A28 | 3593 | 12056 | A33 | CP_NI 27 | L1 | C91 | 2630 | 933 |
| | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h |
| ***Bifidobacterium adolescentis*** | | | | | | | | | | | | | | | | | |
| MB 10/1 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 10 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 |
| MŠ B2 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MŠ B3 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium animalis*** | | | | | | | | | | | | | | | | | |
| BB12 | 6 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 |
| BN | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 10 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 |
| *subsp. lactis* DSM 10140 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 6 | 6 |
| DANONE | 7 | 7 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Nestlè | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MUP 74/7b | 8 | 6 | 6 | 6 | 8 | 6 | 6 | 8 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 |
| D15 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| B28 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 9 | 6 | 6 | 9 | 6 | 6 | 11 | 6 | 6 | 6 |
| B46 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 12 | 6 | 6 | 6 |
| ***Bifidobacterium bifidum*** | | | | | | | | | | | | | | | | | |
| MA1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 11 | 6 | 6 | 6 | 6 | 6 |
| (TMT) NUTRA BONA | 15 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| NORF 78/9 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| E13y | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 12 | 6 |
| ***Bifidobacterium breve*** | | | | | | | | | | | | | | | | | |
| BR03 probiotics drops | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MUP 78/7B | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 8 | 6 | 6 | 11 | 6 | 6 | 6 |
| J19 | 14 | 9 | 6 | 6 | 7 | 6 | 6 | 6 | 11 | 6 | 6 | 8 | 6 | 9 | 6 | 6 | 6 |
| J41 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 12 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| B42 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 11 | 6 | 6 | 6 |
| MUP 77/7a | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 9 | 6 | 6 | 11 | 6 | 6 | 6 |
| NORF 78/9 | 6 | 18 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium catenulatum*** | | | | | | | | | | | | | | | | | |
| DSM 16992 | 6 | 6 | 6 | 9 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| D16 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium pseudocatenulatum*** | | | | | | | | | | | | | | | | | |
| MOTOL 1/8A | 7 | 18 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium longum*** | | | | | | | | | | | | | | | | | |
| NORF 79/8A | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 13 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium pseudolongum*** | | | | | | | | | | | | | | | | | |
| E43 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| BLON | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 |

Low antimicrobial activity (7 – 8 mm)

Medium antimicrobial activity (9 – 13 mm) mm)

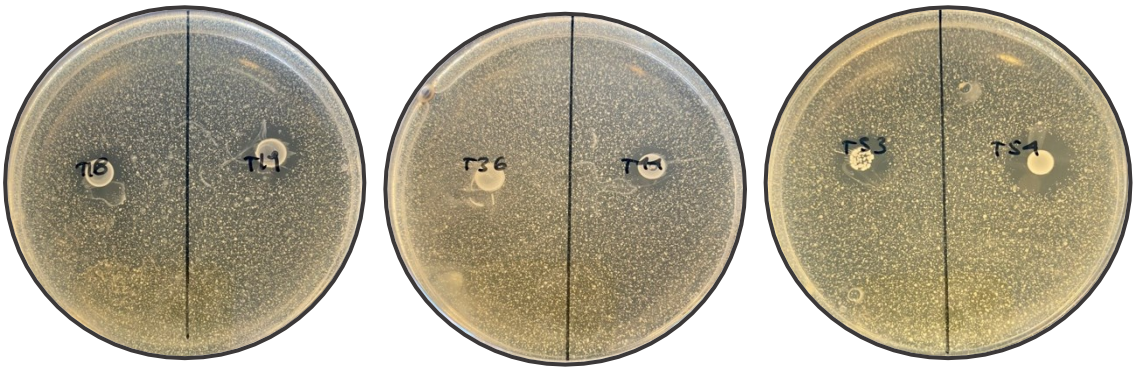
High antimicrobial activity (> 13 mm)

### 3.2.2 Agar spot test

Table 4 describes the results collected with the agar spot test after 48 h of incubation at 37°C in anaerobic conditions. The results are classified in the following ranges: from 7 mm to 10 mm, from 11 mm to 15 mm, and higher than 15 mm.

The largest inhibition zones belonged to *B. catenulatum* D16, isolated from infants, against *C. perfringens* C68 (30 mm), and *B. pseudocatenulatum* MOTOL 1/8A, isolated from adults, against *C. difficile* 3593 (28 mm). Additionally, both of these potential probiotics had antimicrobial activity against *C. butyricum* A74 and *C. difficile* A28, which were isolated from infants' faecal samples. *B. catenulatum* D16 had also effect against *C. paraputrificum* C91 with a 21 mm inhibition halo.

Although *B. catenulatum* D16 and *B. pseudocatenulatum* MOTOL 1/8A reported the highest breadth values, the results clearly show how *B. animalis* is the species with one of the best antimicrobial potential, as evidenced by multiple inhibition zones ranging from 9 to 26 mm. Especially, among *B. animalis* strains investigated in this study, 6 (BN, subsp. *lactis* DSM 10140, Nestlé, D15, B28, and B46) showed antimicrobial activity all clostridial strains tested, apart from *C. butyricum* CP_NI 18, *C. perfringes* 11778, *C. difficile* 12056, and *C. tertium* A33 (Fig.6).




(a) (b) (c)

**Figure 6.** Agar spot test. On the first layer of WSP Agar 1 µl of bifidobacteria was spotted, while the second layer of Anaerobe Basal Agar was homogenized with 40 µl of *Clostridium* spp. cultures. (a) Both *Bifidobacterium animalis* BN (T18) and subsp. *lactis* 10140 (T19) showed antimicrobial activity against *Clostridium perfringes* CP_NI17. (b) *Clostridium perfringes* CP_NI17 growth was inhibited by Bifidobacterium animalis D15 (T44), whereas *Bifidobacterium breve* J41 (T36) did not have any effect (c) Both *Bifidobacterium breve* MUP 77/7a (T53) and *Bifidobacterium breve* MUP 78/7B (T54) had inhibitory effects on *Clostridium perfringes* CP_NI17 growth.

Together with *B. animalis*, *B. breve* exhibited a good ability to decrease clostridial growth: BRE03 probiotic drops, isolated from probiotic products, was positively interacting against 9 distinct strains of clostridia, with stronger results against *C. butyricum* A74 (22 mm inhibitory halo) and *C. difficile* 3593 (25 mm inhibitory halo). Moreover, other *B. breve* strains (MUP 78/7B, B42, and MUP 77/7a), isolated from human samples, had remarkable outcomes anew against *C. butyricum* A74 and CP_NI 11, *C. perfringens* CP_NI17 and *C. difficile* A28, producing inhibitory zones' diameters till 27 mm.

No difference greater than previously described was observed with *B. adolescentis*, *B. longum*, and *B. pseudocatenulatum*, which manifest inhibition halos' diameters only up to 20 mm against a few *Clostridium* spp.

**Table 4**. Inhibitory activity of *Bifidobacterium* spp. against *Clostridium* spp. based on the agar spot test.

| Potential Probiotcs | *Clostridium butyricum* | | | | *Clostridium perfringens* | | | | *Clostridium difficile* | | | *Clostridium tertium* | | *Clostridium neonatale* | *Clostridium paraputrificum* | | *Clostridium clostridioforme* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A74 | 10702 | CP_NI 11 | CP_NI 18 | C68 | 11778 | CP_NI 14 | CP_NI 17 | A28 | 3593 | 12056 | A33 | CP_NI 27 | L1 | C91 | 2630 | 933 |
| | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h |
| **Bifidobacterium adolescentis** | | | | | | | | | | | | | | | | | |
| MB 10/1 | 15 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 14 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| MŠ B2 | 15 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| MŠ B3 | 17 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 15 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| ***Bifidobacterium animalis*** | | | | | | | | | | | | | | | | | |
| BB12 | 9 | 12 | 9 | 6 | 10 | 6 | 6 | 16 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| BN | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 10 | 13 | 25 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| *subsp. lactis* DSM 10140 | 6 | 14 | 6 | 6 | 6 | 6 | 6 | 12 | 14 | 25 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| DANONE | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 11 | 6 | 6 | 6 | 6 | 6 | No detect | 9 | 6 | No detect |
| Nestlé | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 12 | 12 | 26 | 6 | 6 | 14 | No detect | 6 | 6 | No detect |
| MUP 74/7b | 6 | 6 | 10 | 6 | 6 | 6 | 7 | 12 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| D15 | 25 | 19 | 14 | 6 | 16 | 6 | 6 | 10 | 13 | 6 | 6 | 6 | 6 | No detect | 6 | 12 | No detect |
| B28 | 20 | 12 | 22 | 6 | 6 | 6 | 7 | 13 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 15 | No detect |
| B46 | 14 | 6 | 15 | 6 | 7 | 6 | 7 | 14 | 6 | 16 | 6 | 6 | 6 | No detect | 6 | 13 | No detect |
| ***Bifidobacterium bifidum*** | | | | | | | | | | | | | | | | | |
| MA1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| (TMT) NUTRA BONA | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 12 | 6 | 6 | 13 | No detect | 6 | 6 | No detect |
| NORF 78/9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 13 | 10 | 12 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| E13y | 6 | 16 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| ***Bifidobacterium breve*** | | | | | | | | | | | | | | | | | |
| BR03 probiotics drops | 22 | 11 | 14 | 6 | 8 | 7 | 10 | 6 | 6 | 25 | 8 | 6 | 15 | No detect | 6 | 6 | No detect |
| MUP 78/7B | 15 | 6 | 22 | 6 | 8 | 7 | 11 | 16 | 15 | 6 | 6 | 6 | 6 | No detect | 6 | 23 | No detect |
| J19 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| J41 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| B42 | 14 | 23 | 15 | 6 | 7 | 6 | 6 | 13 | 15 | 15 | 6 | 6 | 6 | No detect | 6 | 15 | No detect |
| MUP 77/7a | 22 | 12 | 16 | 6 | 6 | 6 | 6 | 13 | 27 | 6 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| NORF 78/9 | 6 | 14 | 14 | 6 | 6 | 6 | 11 | 6 | 10 | 10 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| **Bifidobacterium catenulatum** | | | | | | | | | | | | | | | | | |
| DSM 16992 | 6 | 18 | 6 | 6 | 6 | 6 | 6 | 14 | 11 | 15 | 6 | 6 | 11 | No detect | 6 | 6 | No detect |
| D16 | 16 | 6 | 6 | 6 | 30 | 6 | 6 | 6 | 17 | 16 | 6 | 6 | 6 | No detect | 21 | 6 | No detect |
| **Bifidobacterium pseudocatenulatum** | | | | | | | | | | | | | | | | | |
| MOTOL 1/8A | 11 | 6 | 6 | 6 | 8 | 6 | 8 | 6 | 18 | 28 | 6 | 6 | 6 | No detect | 6 | 6 | No detect |
| ***Bifidobacterium longum*** | | | | | | | | | | | | | | | | | |
| NORF 79/8A | 6 | 20 | 8 | 6 | 10 | 6 | 6 | 6 | 12 | 6 | 6 | 6 | 20 | No detect | 9 | 6 | No detect |
| ***Bifidobacterium pseudolongum*** | | | | | | | | | | | | | | | | | |
| E43 | 10 | 6 | 6 | 6 | 10 | 6 | 7 | 6 | 9 | 20 | 6 | 6 | 6 | No detect | 15 | 6 | No detect |
| BLON | 13 | 6 | 6 | 6 | 11 | 6 | 6 | 6 | 6 | 6 | 6 | 12 | 6 | No detect | 11 | 6 | No detect |

Low antimicrobial activity (7 – 10 mm)  Medium antimicrobial activity (11 – 15 mm)  High antimicrobial activity (> 15 mm

### 3.2.3 Inhibition of gas production

Then, the final phase of the study focused on the possible probiotic ability to reduce gas generation during a 24-hour period of anaerobic culture. Data from Table 5 can be interpreted as follow: a positive result (+) was used when the inhibition occurred; the mark +ª was used contingent upon a partial inhibition; last, a negative mark (-) was related to the absence of inhibition. A closer inspection of the table shows how *B. animalis susp. lactis* DMS 10140 had a strong effect against both *C. neonatale* L1 and *C. clostridioforme* 933 (Fig. 7 (a), (b); red indicator), and together with *B. breve* J19 against *C. difficile* 3593, thereby affirming as the best bifidobacteria inhibiting gas production. Moreover, the latter bifidobacteria showed also effect against *C. butyricum* CP_NI 18 in a manner that is comparable to *B. adolescentis* MB 10/1 (Fig. 7 (c); red indicator). In addition to *B. animalis susp. lactis* DMS 10140 against *C. difficile* 3593, this pathogen was susceptible to inhibition by *B. animalis* DANONE and *B. bifidum* E13. Other positive results were showed by clostridial strains *C. perfringens* CP_NI 17 and *C. paraputrificum* C91, which were inhibited by *B. pseudocatenulatum* MOTOL 1/8A and *B. pseudolongum* E43, respectively.

The next results, therefore, move on to discuss the partial inhibition of *Clostridium* spp. What is interesting about the data in this table is that *C. butyricum* CP_NI 18 is inhibited by *B. breve* NORF 78/9, *B. pseudocatenulatum* MOTOL 1/8A and by 4 out of 9 *B. animalis* strains (BB12, BN, DANONE, and Nestlé) (Fig. 7 (c); black indicator). Among the *C. butyricum* strains investigated in this study, one more only (A74) got affected by *Bifidobacterial* spp., showing partial results by *B. animalis* Nestlé and *B. pseudolongum* BLON.

*Clostridium neonatale* showed partial results in a specific manner associated with *B. adolescentis* MŠ B2, *B. animalis* BN, *B. animalis* B28, *B. breve* BR03 probiotics drops, and *B. breve* NORF 78/9 (Fig. 7 (d); black indicator). Even though *C. tertium* had not reported any positive result, it was sensitive to the action of 5 different bifidobacterial strains (*B. adolescentis* MŠ B2, *B. adolescentis* MŠ B3, *B. animalis* BN, *B. animalis* DANONE, and *B. pseudolongum* BLON). Other partial inhibitions were also collected as shown for *C.*

*perfringens* 11778, *C. perfringens* CP_NI17, *C. difficile* A28, *C. difficile* 3593, and *C. clostridioforme* 933.

Eventually, *C. paraputrificum* showed itself as the most resistant clostridia, apart from the inhibition from *B. pseudolongum* E43.

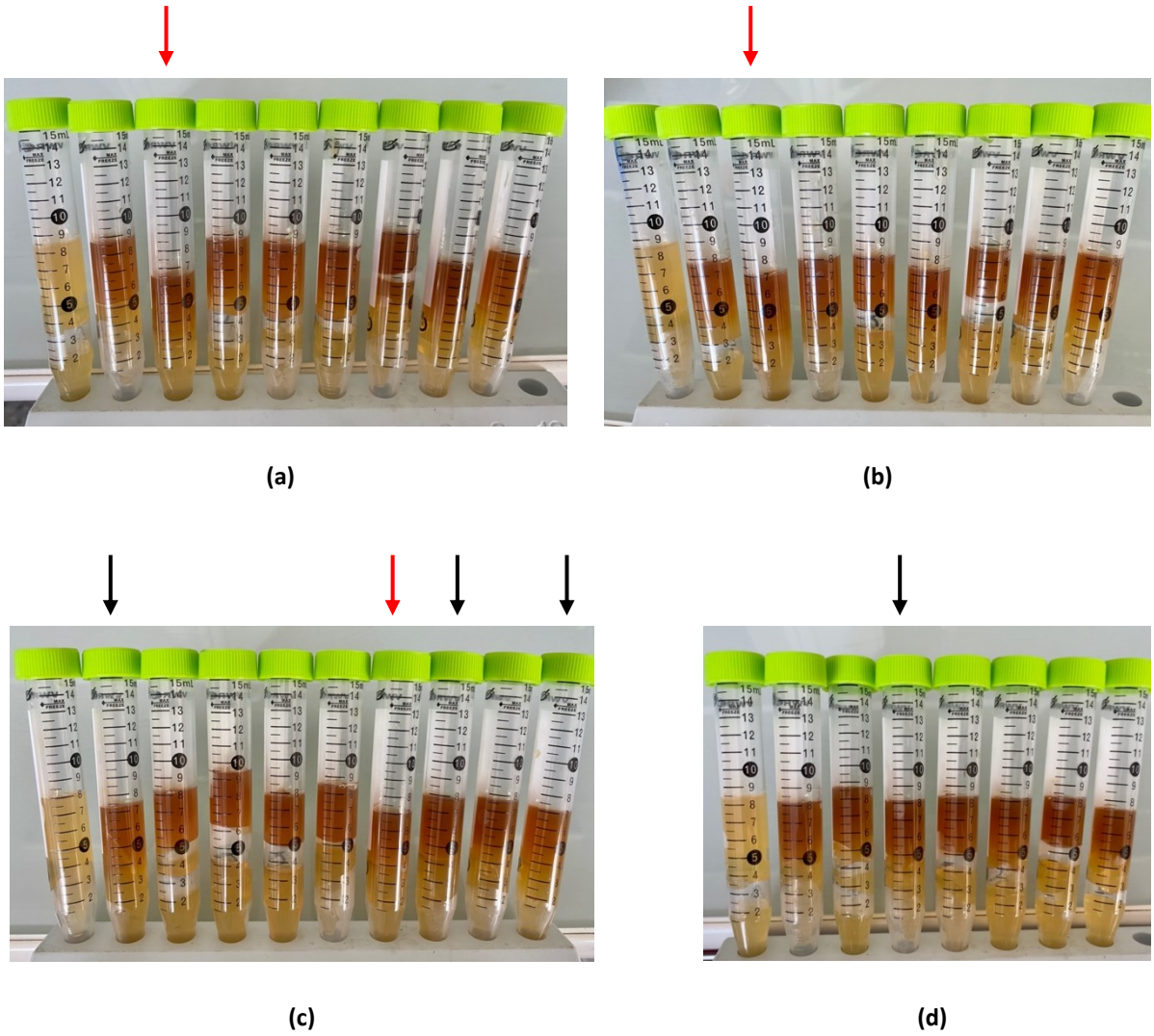



**Figure 7.** Inhibition of gas production assay. The lower layer corresponds to the Clostridium difficile Agar inoculated with 600 µl overnight culture of Clostridium spp. and the second layer is MRS Agar inoculated with 120 µl bifidobacterial culture. (a) *Bifidobacterium animalis susp. lactis* DMS 10140 completely suppressed *Clostridium neonatale* L1 from gas production (red indicator). (b) *Clostridium clostridioforme* 933 gas generation was completely stopped by *Bifidobacterium animalis susp. lactis* DMS 10140 (red indicator). (c) *Clostridium butyricum* CP_NI18 gas production was inhibited both totally by *Bifidobacterium adolescentis* MB 10/1 (red indicator) and partially by *Bifidobacterium pseudocatenulatum* MOTOL 1/8A, *Bifidobacterium animalis* BB12, and *Bifidobacterium animalis* BN (black indicator). (d) *Bifidobacterium breve* BR03 probiotic drops partially suppressed the generation of gas by *Clostridium neonatale* L1 (black indicator).

**Table 5** Inhibition of gas production essay.

| Potential probiotics | *Clostridium butyricum* | | | | *Clostridium perfringens* | | | | *Clostridium difficile* | | | *Clostridium tertium* | | *Clostridium neonatale* | *Clostridium paraputrificum* | | *Clostridium clostridioforme* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A74 | 10702 | CP_NI 11 | CP_NI 18 | C8 | 11778 | CP_NI 14 | CP_NI 17 | A28 | 3593 | 12056 | A33 | CP_NI 27 | L1 | C91 | 2630 | 933 |
| | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h | 48h |
| **Bifidobacterium adolescentis** | | | | | | | | | | | | | | | | | |
| MB 10/1 | - | - | - | + | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| MŠ B2 | - | - | - | - | - | - | No detect | - | - | - | - | +ª | - | +ª | - | - | +ª |
| MŠ B3 | - | - | - | - | - | - | No detect | - | - | - | - | +ª | - | - | - | - | - |
| ***Bifidobacterium animalis*** | | | | | | | | | | | | | | | | | |
| BB12 | - | - | - | +ª | - | +ª | No detect | - | +ª | - | - | - | - | - | - | - | - |
| BN | - | - | - | +ª | - | - | No detect | - | - | - | - | +ª | - | +ª | - | - | - |
| *subsp. lactis* DSM 10140 | - | - | - | - | - | - | No detect | - | - | + | - | - | - | + | - | - | + |
| DANONE | - | - | - | +ª | - | - | No detect | - | - | + | - | +ª | - | - | - | - | - |
| Nestlé | +ª | - | - | +ª | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| MUP 74/7b | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| D15 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | +ª |
| B28 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | +ª | - | - | - |
| B46 | - | - | - | - | - | - | No detect | +ª | - | - | - | - | - | - | - | - | - |
| ***Bifidobacterium bifidum*** | | | | | | | | | | | | | | | | | |
| MA1 | + | - | - | - | - | - | No detect | - | +ª | +ª | - | - | - | - | - | - | - |
| (TMT) NUTRA BONA | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| NORF 78/9 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| E13y | - | - | - | - | - | - | No detect | - | - | + | - | - | - | - | - | - | - |
| ***Bifidobacterium breve*** | | | | | | | | | | | | | | | | | |
| BR03 probiotics drops | - | - | - | - | - | - | No detect | - | +ª | +ª | - | - | - | +ª | - | - | +ª |
| MUP 78/7B | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| J19 | - | - | - | + | - | - | No detect | - | - | - | - | - | - | + | - | - | - |
| J41 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| B42 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| MUP 77/7a | - | - | - | - | - | - | No detect | +ª | - | - | - | - | - | - | - | - | - |
| NORF 78/9 | - | - | - | +ª | - | - | No detect | - | - | - | - | - | - | +ª | - | - | - |
| **Bifidobacterium catenulatum** | | | | | | | | | | | | | | | | | |
| DSM 1-992 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| D16 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| **Bifidobacterium pseudocatenulatum** | | | | | | | | | | | | | | | | | |
| MOTOL 1/8A | - | - | - | +ª | - | - | No detect | + | - | - | - | - | - | - | - | - | - |
| ***Bifidobacterium longum*** | | | | - | | | | | | | | | | | | | |
| NORF 79/8A | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | - | - | - |
| ***Bifidobacterium pseudolongum*** | | | | | | | | | | | | | | | | | |
| E43 | - | - | - | - | - | - | No detect | - | - | - | - | - | - | - | + | - | - |
| BLON | +ª | - | - | - | - | - | No detect | +ª | - | - | - | +ª | - | - | - | - | - |

**(+)** Positive: inhibition occured **(+ª)** Partial inhibition **(-)** Negative: inhibition not occured

## 3.2 Comparative analysis between the diffusion and the agar spot method

The inhibition exacerbated by potential probiotics is comparable between the diffusion method and the agar spot test since their results are based on similar activities. Surprisingly, during the analysis are being highlighted notable similarities as well as differences ones and the most relevant ones are marked in green and red, respectively, in the table below (Tab. 6). Particularly, the differences were only taken into consideration when the difference in halo diameters between the two methods was more than 8 mm. Comparisons with *C. neonatale* L1 and *C. clostridioforme* 933 were not performed, due to laboratory complications, that prevented from reporting the results of the agar spot test.

***Similarities***

The Table 6 reveals how 2 out of 4 *C. butyricum* strains showed inhibition halos in both protocols: A74 was inhibited by *B. animalis* DANONE and B*. pseudocatenulatum* MOTOL 1/8A, while 10702 was inhibited by *B. bifidum* E13y and *B. breve* (BR03 probiotics drops, NORF 78/9). In like manner, *C. perfringens* CP_NI 17 shared inhibitions from the strains *B. animalis* BN and *B. breve* MUP 77/7a. Additionally, the growth of *C. tertium* strains (A33 and CP_NI27) was reduced after the effect of *B. pseudolongum* BLON and *B. longum* NORF 79/8A, respectively. The last significant similarity is referrable to *C. difficile* A28 treated with *B. breve* B42.

***Differences***

Shifting the focus from similarities (green mark) to differences (red mark), the effects of *Bifidobacterium catenulatum* D16 are what make the data in this table intriguing. *C. butyricum* A74, *C. perfringens* C68, *C. difficile* A28, C*. difficile* 3593, and C*. paraputrificum* C91 appeared resistant to this potential probiotic since no inhibition halos were compared with the diffusion method. However, inhibitory zones with diameters greater than 15 mm were reported using the agar spot test. An analogous condition occurred with *B. breve* MUP 78/7B against *C. butyricum* A74 and CP_NI11, *C. perfringens* CP_NI17, *C. difficile* A28 and *C. paraputrificum* 2630. None of the *B. adolescentis* strains tested

against *C. butyricum* A74 produced any inhibitory halo when evaluated using the diffusion method, whereas the agar spot test resulted in a reduction of this pathogen growth, with inhibitory halo diameters ranging from 15 to 17 mm. Similar to this, no one *B. animalis* strains exhibited antimicrobial activity against *C. difficile* 3593 in the diffusion method, but 4 out of 9 strains (BN, subsp. *lactis* DSM 10140, Nestlé and B46) inhibited pathogen growth, with inhibitory halo diameters ranging from 16 to 26 mm.

Even though the best inhibitory halos compared using the agar spot test, trend reversal occurred with *B. bifidum* (TMT) NUTRA BONA against *C. butyricum* A74 and *B. pseudocatenulatum* MOTOL 1/8 against *C. butyricum* 10702. The agar spot test yielded negative findings in both instances; however, the diffusion method revealed a decrease in pathogen growth with inhibitory halos of 15 and 18 mm, respectively.

**Table 6**. Comparative analysis between the diffusion method and the agar spot method.
DM: diffusion method AST: agar spot test

| Potential Probiotics | Diameter of Inhibition Zones (mm) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Clostridium butyricum* | | | | | | | | *Clostridium perfringens* | | | | | | | |
| | A74 | | 10702 | | CP_NI 11 | | CP_NI 18 | | C68 | | 11778 | | CP_NI 14 | | CP_NI 17 | |
| | DM | AST | DM | AST | DM | AST | DM | AST | DM | AST | DM | AST | DM | AST | DM | AST |
| **Bifidobacterium adolescentis** | | | | | | | | | | | | | | | | |
| MB 10/1 | 6 | 15 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 10 | 6 |
| MŠ B2 | 6 | 15 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 |
| MŠ B3 | 6 | 17 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium animalis*** | | | | | | | | | | | | | | | | |
| BB12 | 6 | 9 | 6 | 12 | 6 | 9 | 10 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 16 |
| BN | 6 | 6 | 6 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 10 | 10 |
| *subsp. lactis* DSM 10140 | 6 | 6 | 6 | 14 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 12 |
| DANONE | 7 | 9 | 7 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 11 |
| Neslè | 6 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 12 |
| MUP 74/7b | 8 | 6 | 6 | 6 | 6 | 10 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 7 | 8 | 12 |
| D15 | 6 | 25 | 6 | 19 | 6 | 14 | 6 | 6 | 6 | 16 | 6 | 6 | 6 | 6 | 6 | 10 |
| B28 | 6 | 20 | 6 | 12 | 6 | 22 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 7 | 9 | 13 |
| B46 | 6 | 14 | 6 | 6 | 6 | 15 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 7 | 6 | 14 |
| ***Bifidobacterium bifidum*** | | | | | | | | | | | | | | | | |
| MA1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| (TMT) NUTRA BONA | 15 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| NORF 78/9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 13 |
| E13y | 6 | 6 | 9 | 16 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 |
| ***Bifidobacterium breve*** | | | | | | | | | | | | | | | | |
| BR03 probiotics drops | 6 | 22 | 7 | 11 | 6 | 14 | 6 | 6 | 6 | 8 | 6 | 7 | 6 | 10 | 6 | 6 |
| MUP 78/7B | 6 | 15 | 6 | 6 | 6 | 22 | 6 | 6 | 6 | 8 | 6 | 7 | 6 | 11 | 8 | 16 |
| J19 | 14 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| J41 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| B42 | 6 | 14 | 6 | 23 | 6 | 15 | 6 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 13 |
| MUP 77/7a | 6 | 22 | 6 | 12 | 6 | 16 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 13 |
| NORF 78/9 | 6 | 6 | 18 | 14 | 6 | 14 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 11 | 6 | 6 |
| **Bifidobacterium catenulatum** | | | | | | | | | | | | | | | | |
| DSM 16992 | 6 | 6 | 6 | 18 | 6 | 6 | 9 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 14 |
| D16 | 6 | 16 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 30 | 6 | 6 | 6 | 6 | 6 | 6 |
| **Bifidobacterium pseudocatenulatum** | | | | | | | | | | | | | | | | |
| MOTOL 1/8A | 7 | 11 | 18 | 6 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 8 | 6 | 6 |
| ***Bifidobacterium longum*** | | | | | | | | | | | | | | | | |
| NORF 79/8A | 6 | 6 | 6 | 20 | 6 | 8 | 6 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium pseudolongum*** | | | | | | | | | | | | | | | | |
| E43 | 6 | 10 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 10 | 6 | 6 | 6 | 7 | 6 | 6 |
| BLON | 6 | 13 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 11 | 6 | 6 | 6 | 6 | 6 | 6 |

Similarities

Differences

**Table 6.** (Continued)

Diameter of Inhibition Zones (mm)

| Potential Probiotics | *Clostridium difficile* | | | | | | *Clostridium tertium* | | | | *Clostridium paraputrificum* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A28 | | 3593 | | 12056 | | A33 | | CP_NI 27 | | C91 | | 2630 | |
| | AST | DM | AST | DM | AST | DM | DM | AST | DM | AST | DM | AST | DM | AST |
| **Bifidobacterium adolescentis** | | | | | | | | | | | | | | |
| MB 10/1 | 6 | 14 | 6 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MŠ B2 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MŠ B3 | 6 | 15 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium animalis*** | | | | | | | | | | | | | | |
| BB12 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| BN | 6 | 13 | 6 | 25 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| *subsp. lactis* DSM 10140 | 6 | 14 | 6 | 25 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 6 | 6 | 6 |
| DANONE | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 6 | 6 |
| Neslè | 6 | 12 | 6 | 26 | 6 | 6 | 6 | 6 | 6 | 14 | 6 | 6 | 6 | 6 |
| MUP 74/7b | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| D15 | 6 | 13 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 12 |
| B28 | 6 | 6 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 15 |
| B46 | 6 | 6 | 6 | 16 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 13 |
| ***Bifidobacterium bifidum*** | | | | | | | | | | | | | | |
| MA1 | 6 | 6 | 6 | 6 | 6 | 6 | 11 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| (TMT) NUTRA BONA | 6 | 6 | 6 | 12 | 6 | 6 | 6 | 6 | 6 | 13 | 6 | 6 | 6 | 6 |
| NORF 78/9 | 6 | 10 | 6 | 12 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| E13y | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 12 | 6 |
| ***Bifidobacterium breve*** | | | | | | | | | | | | | | |
| BR03 probiotics drops | 8 | 6 | 6 | 25 | 6 | 8 | 6 | 6 | 6 | 15 | 6 | 6 | 6 | 6 |
| MUP 78/7B | 6 | 15 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 23 |
| J19 | 11 | 6 | 6 | 6 | 6 | 6 | 8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| J41 | 12 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| B42 | 8 | 15 | 6 | 15 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 15 |
| MUP 77/7a | 6 | 27 | 6 | 6 | 9 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| NORF 78/9 | 6 | 10 | 6 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| **Bifidobacterium catenulatum** | | | | | | | | | | | | | | |
| DSM 16992 | 6 | 11 | 6 | 15 | 6 | 6 | 6 | 6 | 6 | 11 | 6 | 6 | 6 | 6 |
| D16 | 6 | 17 | 6 | 16 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 21 | 6 | 6 |
| **Bifidobacterium pseudocatenulatum** | | | | | | | | | | | | | | |
| MOTOL 1/8A | 6 | 18 | 6 | 28 | 6 | 6 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| ***Bifidobacterium longum*** | | | | | | | | | | | | | | |
| NORF 79/8A | 6 | 12 | 6 | 6 | 6 | 6 | 6 | 6 | 13 | 20 | 6 | 9 | 6 | 6 |
| ***Bifidobacterium pseudolongum*** | | | | | | | | | | | | | | |
| E43 | 6 | 9 | 6 | 20 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 15 | 6 | 6 |
| BLON | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 12 | 6 | 6 | 6 | 11 | 6 | 6 |

Similarities

Differences

# 4. DISCUSSION

## 4.1 Evaluation of the antimicrobial activity of bifidobacteria against clostridia

The results of this study indicate how *Bifidobacterium* spp. can show antimicrobial activity against clostridial species. The bacteria's antimicrobial activity can be translated on their ability to produce metabolites which contribute to the effective decrease of pathogen viability as well as their metabolism. Among pathogenetic bacteria, clostridia are mostly implicated in the etiology of infant diseases, such as necrotizing enterocolitis and antibiotic-associated diarrhea.

This study has not only been focused on species already observed in the literature such as *Clostridium butyricum*, *Clostridium perfringens* and *Clostridioides difficile*, but also *Clostridium tertium*, *Clostridium neonatale*, *Clostridium paraputrificum*, and *Enterocloster clostridioformis*. Originally these species belonged to *Clostridium* genus, *Clostridiaceae* family, and *Firmicutes* phylum. However molecular techniques revealed the extensive phylogenetic variety of the genus *Clostridium*, especially phylogenetic analysis based on 16S rRNA gene sequencing demonstrated that a few species are included in different families. According to Lawson et al., 2016a *Clostridium difficile* is located in *Peptostreptococcaceae* family and it is reclassified as *Clostridioides difficile,* although the original name might be still used. Similarly, genomic, phenotypic and ecologic features aided in the differentiation of *Clostridium clostridioforme* species: this pathogen is included in *Lachnospiraceae* family and it is reclassified as *Enterocloster clostridioformis*, as proposed by Haas & Blanchard, 2020.

The current treatment of infectious intestinal disorders is based on the use of antibiotics, such as vancomycin and fidaxomicin, although infections may occur after antibiotics treatments (Jarmo et al., 2020). Since antibiotic medication has a negative impact on the composition and metabolic activity of the gut microbiota, research on probiotics are being developed for the prevention and treatment of clostridia infections (Mills et al., 2018). Among all potential probiotics, bifidobacteria are frequently utilized as probiotics in newborn infants for both therapeutic and preventative purposes because of their high prevalence in the GIT tract and capacity to colonize the gut (di Gioia et al., 2014).

In this study bifidobacteria were investigated in order to discover their antimicrobial activity against different species of clostridia. Their ability to inhibit pathogen's growth is connected to bacterial fermentation resulting in short-chain fatty acids, such as acetate, butyrate and propionate (Dürre, 2014).

In terms of inhibition of clostridia's growth, the majority of *Bifidobacterium breve* and *Bifidobacterium animalis* strains tested in this study had the greatest results against *Clostridium butyricum*, *Clostridium perfringes* and *Clostridium difficile*. These findings are supported by similar ones, such as the investigation from Schoster et al., 2013 that proved the inhibitory effect of *B. animalis* subsp. *lactis* against both *C. perfringens* and *C. difficile*. Moreover, the latter one might be fully inhibited by *B. breve,* mainly by interfering negatively with physiological processes and causing damage on cell morphology (Sorg et al., 2019).

Although mostly of results are in agreement with the literature there are some controversial results, especially among *B. animalis* strains. According to Merenstein et al., 2021, *B. animalis* BB12 which is one of the most documented probiotic *Bifidobacterium*, should protect from *C. difficile* infections. Surprisingly, this study did not detect any inhibitory halos either with diffusion method or agar spot method; additionally, no great inhibition of gas was detected.

It is interesting how not all of the studied bacterial species have an equal representation of strains. Due to this, the outcomes from *Bifidobacterium longum*, which had just one strain, could not be considered as significant as the results obtained from *B. animalis* and *B. breve*, which were represented by nine and seven strains, respectively.

Comparing between agar spot test and diffusion method for the width of the inhibition reveals that the second method provided the best inhibitory halos. Even though the agar spot test yields the greatest outcomes, some data were missing with this method due to laboratory problematics related to the *Clostridium* spp. growing conditions. The variability in performance suggests that more than one method should be used to properly assess the antimicrobial activity of potential probiotics, considering that the condition of each approach may affect the results.

## 4.2 Bifidobacteria's antimicrobial activity is both species and strains specific

Interestingly, most of the bifidobacteria tested generally exhibit antimicrobial activity against clostridia, in particular *Clostridium neonatale* L1 reported a good inhibition both by *Bifidobacterium breve* (MUP 78/7B, J19, B42, and MUP 77/7a) and *Bifidobacterium animalis* (BN, subsp. *lactis* DSM 10140, B28, and B46) when tested with diffusion method and inhibition of gas production. However, not all the strains belonging to a specie exhibit the same effect against clostridia and this observation may support the hypothesis that antimicrobial activity is both species and strains specific. The heterogeneity in their capacity to prevent clostridial growth might be referred to the various origins of the strains. Tested bifidobacterial strain originated from infants and adult's faeces, and some were isolated from probiotic or dairy products. Since the microbiota is influenced by different factors, such as delivery mode and type of feeding as documented in several studies, all the strains can exhibit different actions against clostridia. In addition, it is proved that the carbon source might affect the interaction of bifidobacteria with intestinal epithelial cells. As showed by Wickramasinghe et al., 2015, bifidobacteria grown on human milk oligosaccharides decreased the level of inflammatory markers, compared to glucose or lactose-produced bacteria. These results provide further support for the hypothesis that breast feeding affects gut microbiota, which in turn influences the antimicrobial activity of bifidobacteria.

These finding strengthens the idea that *Bifidobacteri*a spp. might be utilized as probiotic supplements for the treatment of clostridial gastrointestinal disorders.
However certain limitations of the study should be taken in consideration. Firstly, apart from secretion of antibacterial substance which determine inhibition of growth, the antimicrobial activity of potential probiotics should be evaluated with other methodologies such as adhesive propeters to host cell and mucin, tolerance to acidic pH and bile salts, nutrient and ecological niche competition, spore and toxin production, and virulence gene expression (Yang & Yang, 2019; Monteiro et al., 2019). Moreover, each species should be represented by a similar number of strains in order to have comparable findings.

# 5. CONCLUSION AND FUTURE PERSPECTIVES

Clostridial infections are responsible of severe gastrointestinal disorders among infants, including necrotizing enterocolitis and antibiotic-associated diarrhea. The present study was designed to determine the antimicrobial activity of bifidobacteria against clostridia. The findings indicated that *Bifidobacterium breve* and *Bifidobacterium animalis* affect different clostridial species, with notable results against *Clostridium neonatale* L1. The inhibitory activity of these bacteria was also validated against *Clostridium perfringes* and *Clostridium difficile* as mentioned in the literature (Schoster et al., 2013; Sorg et al., 2019).

The findings allow to conclude that bifidobacteria might work as probiotics with a positive impact on infant health, however further studies are required to evaluate their probiotic potential, including an analysis of coaggregation ability, adhesion properties of mucin and host cells, tolerance to acidic pH, and bile salt tolerance.

Nevertheless, additional clinical research should be undertaken to explore how carbon source might influence the antimicrobial activity of bifidobacteria in infants GIT; future preventative or therapeutic probiotic supplement formulations could need to incorporate carefully selected prebiotics (Wickramasinghe et al., 2015).

# BIBLIOGRAFY


Adak, A., Mojibur, ·, & Khan, R. (2019). *An insight into gut microbiota and its functionalities*. *76*, 473–493. https://doi.org/10.1007/s00018-018-2943-4

Andrade, J. C., Almeida, D., Domingos, M., Seabra, C. L., Machado, D., Freitas, A. C., & Gomes, A. M. (2020). Commensal obligate anaerobic bacteria and health: Production, storage, and delivery strategies. In *Frontiers in Bioengineering and Biotechnology* (Vol. 8, pp. 1–23). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2020.00550

Arboleya, S., Ang, L., Margolles, A., Yiyuan, L., Dongya, Z., Liang, X., Solís, G., Fernández, N., de los Reyes-Gavilán, C. G., & Gueimonde, M. (2012). Deep 16S rRNA metagenomics and quantitative PCR analyses of the premature infant fecal microbiota. *Anaerobe*, *18*(3), 378–380. https://doi.org/10.1016/j.anaerobe.2012.04.013

Arboleya, S., Sánchez, B., Milani, C., Duranti, S., Solís, G., Fernández, N., de Los Reyes-Gavilán, C. G., Ventura, M., Margolles, A., & Gueimonde, M. (2015). Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *Journal of Pediatrics*, *166*(3), 538–544. https://doi.org/10.1016/j.jpeds.2014.09.041

Ariyoshi, T., Hagihara, M., Takahashi, M., & Mikamo, H. (2022). Effect of Clostridium butyricum on Gastrointestinal Infections. In *Biomedicines* (Vol. 10, Issue 2). MDPI. https://doi.org/10.3390/biomedicines10020483

Arrieta, M.-C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., Finlay, B., Massari, P., & Strunk, T. (2014). *The intestinal microbiome in early life: health and disease*. https://doi.org/10.3389/fimmu.2014.00427

Attebery, H. R., & Finegold, S. M. (1969). Combined Screw-Cap and Rubber-Stopper Closure for Hungate Tubes (Pre-reduced Anaerobically Sterilized Roll Tubes and Liquid Media). In *APPLIED MICROBIOLOGY*. https://journals.asm.org/journal/am

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/J.JPHA.2015.11.005

Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C., & Schmidt, T. M. (2019). Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. *MBio*, *10*(1). https://doi.org/10.1128/mBio.02566-18

Binetti, A. G., Gómez, E. M., Pulgar, D., Fiocco, D., Ouwehand, A. C., Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., & Tremblay, A. (2020). *Criteria to Qualify Microorganisms as ``Probiotic'' in Foods and Dietary Supplements*. https://doi.org/10.3389/fmicb.2020.01662

Cassir, N., Benamar, S., & la Scola, B. (2016). Clostridium butyricum: From beneficial to a new emerging pathogen. In *Clinical Microbiology and Infection* (Vol. 22, Issue 1, pp. 37–45). Elsevier B.V. https://doi.org/10.1016/j.cmi.2015.10.014

di Gioia, D., Aloisio, I., Mazzola, G., & Biavati, B. (2014). Bifidobacteria: Their impact on gut microbiota composition and their applications as probiotics in infants. In *Applied Microbiology and Biotechnology* (Vol. 98, Issue 2, pp. 563–577). https://doi.org/10.1007/s00253-013-5405-9

Dürre, P. (2014). *Physiology and Sporulation in Clostridium*. https://doi.org/10.1128/microbiolspec.TBS-0010-2012

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J. M., Topping, D. L., Suzuki, T., Taylor, T. D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M., & Ohno, H. (2011). *Bifidobacteria can protect from enteropathogenic infection through production of acetate*. https://doi.org/10.1038/nature09646

Golić, N., Veljović, K., Popović, N., Djokić, J., Strahinić, I., Mrvaljević, I., & Terzić-Vidojević, A. (2017). In vitro and in vivo antagonistic activity of new probiotic culture against Clostridium difficile and Clostridium perfringens. *BMC Microbiology*, *17*(1), 1–9. https://doi.org/10.1186/S12866-017-1015-5/FIGURES/5

Gomaa, E. Z. (n.d.). Human gut microbiota/microbiome in health and diseases: a review. *Antonie van Leeuwenhoek*, *113*. https://doi.org/10.1007/s10482-020-01474-7

Gomaa, E. Z. (2020). Human gut microbiota/microbiome in health and diseases: a review. *Antonie van Leeuwenhoek*, *113*(12), 2019–2040. https://doi.org/10.1007/s10482-020-01474-7

Haas, K. N., & Blanchard, J. L. (2020). Reclassification of the clostridium clostridioforme and clostridium sphenoides clades as enterocloster gen. Nov. and lacrimispora gen.

nov., including reclassification of 15 taxa. *International Journal of Systematic and Evolutionary Microbiology*, *70*(1), 23–34. https://doi.org/10.1099/ijsem.0.003698

Hooper, L. v, & Macpherson, A. J. (2015). *Microbiota Immune adaptations that maintain homeostasis with the intestinal microbiota*. https://doi.org/10.1038/nri2710

Jarmo, O., Veli-Jukka, A., & Eero, M. (2020). Treatment of *Clostridioides (Clostridium) difficile* infection. *Annals of Medicine*, *52*(1–2), 12–20. https://doi.org/10.1080/07853890.2019.1701703

Jungersen, M., Wind, A., Johansen, E., Christensen, J. E., Stuer-Lauridsen, B., & Eskesen, D. (2014). The science behind the probiotic strain bifidobacterium animalis subsp. Lactis bb-12®. In *Microorganisms* (Vol. 2, Issue 2, pp. 92–110). MDPI AG. https://doi.org/10.3390/microorganisms2020092

Lawson, P. A., Citron, D. M., Tyrrell, K. L., & Finegold, S. M. (2016). Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. *Anaerobe*, *40*, 95–99. https://doi.org/10.1016/J.ANAEROBE.2016.06.008

Meister, A. L., Doheny, K. K., & Travagli, R. A. (2020). Necrotizing enterocolitis: It's not all in the gut. *Experimental Biology and Medicine*, *245*(2), 85–95. https://doi.org/10.1177/1535370219891971

Merenstein, D., Fraser, C. M., Roberts, R. F., Liu, T., Grant-Beurmann, S., Tan, T. P., Smith, K. H., Cronin, T., Martin, O. A., Sanders, M. E., Lucan, S. C., & Kane, M. A. (2021a). Bifidobacterium animalis subsp. Lactis bb-12 protects against antibiotic-induced functional and compositional changes in human fecal microbiome. *Nutrients*, *13*(8). https://doi.org/10.3390/nu13082814

Merenstein, D., Fraser, C. M., Roberts, R. F., Liu, T., Grant-Beurmann, S., Tan, T. P., Smith, K. H., Cronin, T., Martin, O. A., Sanders, M. E., Lucan, S. C., & Kane, M. A. (2021b). Bifidobacterium animalis subsp. lactis BB-12 Protects against Antibiotic-Induced Functional and Compositional Changes in Human Fecal Microbiome. *Nutrients*, *13*(8), 2814. https://doi.org/10.3390/nu13082814

Mills, J. P., Rao, K., & Young, V. B. (2018). Probiotics for prevention of Clostridium difficile infection. *Current Opinion in Gastroenterology*, *34*(1), 3–10. https://doi.org/10.1097/MOG.0000000000000410

Modesto, M., Checcucci, A., & Mattarelli, P. (2021). *Identification of Bifidobacteria by the Phosphoketolase Assay* (pp. 141–148). https://doi.org/10.1007/978-1-0716-1274-3_12

Monteiro, C. R. A. V., do Carmo, M. S., Melo, B. O., Alves, M. S., dos Santos, C. I., Monteiro, S. G., Bomfim, M. R. Q., Fernandes, E. S., & Monteiro-Neto, V. (2019). In vitro antimicrobial activity and probiotic potential of bifidobacterium and lactobacillus against species of clostridium. *Nutrients*, *11*(2). https://doi.org/10.3390/nu11020448

Nagai, M., Obata, Y., Takahashi, D., & Hase, K. (2016). Fine-tuning of the mucosal barrier and metabolic systems using the diet-microbial metabolite axis. *International Immunopharmacology*, *37*, 79–86. https://doi.org/10.1016/j.intimp.2016.04.001

Normand, A. C., Cassagne, C., Gautier, M., Becker, P., Ranque, S., Hendrickx, M., & Piarroux, R. (2017). Decision criteria for MALDI-TOF MS-based identification of filamentous fungi using commercial and in-house reference databases. *BMC Microbiology*, *17*(1), 1–17. https://doi.org/10.1186/s12866-017-0937-2

Parte, A. C., Sardà Carbasse, J., Meier-Kolthoff, J. P., Reimer, L. C., & Göker, M. (2020). List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *International Journal of Systematic and Evolutionary Microbiology*, *70*(11), 5607–5612. https://doi.org/10.1099/ijsem.0.004332

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van den Brandt, P. A., & Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, *118*(2), 511–521. https://doi.org/10.1542/peds.2005-2824

Pokusaeva, K., Fitzgerald, G. F., & van Sinderen, D. (2011). Carbohydrate metabolism in Bifidobacteria. *Genes & Nutrition*, *6*(3), 285–306. https://doi.org/10.1007/s12263-010-0206-6

Popoff, M. R., & Bouvet, P. (2013). Genetic characteristics of toxigenic Clostridia and toxin gene evolution. *Toxicon*, *75*, 63–89. https://doi.org/10.1016/j.toxicon.2013.05.003

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Abele, G., Miggiano, D., Gasbarrini, A., & Mele, M. C. (2019). *microorganisms What is the Healthy Gut Microbiota*

*Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases*. *7*, 14. https://doi.org/10.3390/microorganisms7010014

Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. In *Nature Reviews Immunology* (Vol. 16, Issue 6, pp. 341–352). Nature Publishing Group. https://doi.org/10.1038/nri.2016.42

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., Tuohy, K., Heinken Almutheinken, A., Karen Scott KScott, unilu, & Jonathan Swann, abdnacuk. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *European Journal of Nutrition*, *57*, 1–24. https://doi.org/10.1007/s00394-017-1445-8

Rui, W., Gu, C., Zhang, H., Liao, X., Zhao, X., Xu, Y., & Yang, J. (2022). Antagonistic activity of selenium-enriched Bifidobacterium breve against Clostridioides difficile. *Applied Microbiology and Biotechnology*, *106*(18), 6181–6194. https://doi.org/10.1007/s00253-022-12124-5

Santos do Carmo, M., itapary dos Santos, C., Calácio Araújo, M., Alberto Girón, J., Soares Fernandes, E., & Monteiro-Neto, V. (2018). *Food & Function REVIEW Probiotics, mechanisms of action, and clinical perspectives for diarrhea management in children*. https://doi.org/10.1039/c8fo00376a

Saturio, S., Nogacka, A. M., Alvarado-Jasso, G. M., Salazar, N., de Los Reyes-Gavilán, C. G., Gueimonde, M., & Arboleya, S. (2021). *microorganisms Role of Bifidobacteria on Infant Health*. https://doi.org/10.3390/microorganisms9122415

Schönherr-Hellec, S., & Aires, J. (2019). Clostridia and necrotizing enterocolitis in preterm neonates. In *Anaerobe* (Vol. 58, pp. 6–12). Academic Press. https://doi.org/10.1016/j.anaerobe.2019.04.005

Schönherr-Hellec, S., Klein, G. L., Delannoy, J., Ferraris, L., Rozé, J. C., Butel, M. J., & Aires, J. (2018). Clostridial strain-specific characteristics associated with necrotizing enterocolitis. *Applied and Environmental Microbiology*, *84*(7). https://doi.org/10.1128/AEM.02428-17

Schoster, A., Kokotovic, B., Permin, A., Pedersen, P. D., Bello, F. D., & Guardabassi, L. (2013). In vitro inhibition of Clostridium difficile and Clostridium perfringens by commercial probiotic strains. *Anaerobe*, *20*, 36–41. https://doi.org/10.1016/j.anaerobe.2013.02.006

Shen, A., Edwards, A. N., Sarker, M. R., & Paredes-Sabja, D. (2019). Sporulation and Germination in Clostridial Pathogens. *Microbiology Spectrum*, *7*(6). https://doi.org/10.1128/microbiolspec.gpp3-0017-2018

Sorg, J., Wheelhouse, N., Yang, H., & Yang, J. (2019). Antibacterial Activity of Bifidobacterium breve Against Clostridioides difficile. *Frontiers in Cellular and Infection Microbiology | Www.Frontiersin.Org*, *1*, 288. https://doi.org/10.3389/fcimb.2019.00288

Tagg, J. R., & Mcgiven, A. R. (1971). Assay System for Bacteriocins. In *APPLIED MICROBIOLOGY* (Vol. 21, Issue 5). https://journals.asm.org/journal/am

Thursby, E., & Juge, N. (2017). *Introduction to the human gut microbiota*. https://doi.org/10.1042/BCJ20160510

Turroni, F., Milani, C., Duranti, S., Ferrario, C., Gabriele, ·, Lugli, A., Mancabelli, L., Douwe Van Sinderen, ·, & Ventura, · Marco. (2018). Bifidobacteria and the infant gut: an example of co-evolution and natural selection. *Cell. Mol. Life Sci*, *75*, 103–118. https://doi.org/10.1007/s00018-017-2672-0

Uzal, F. A., Freedman, J. C., Shrestha, A., Theoret, J. R., Garcia, J., Awad, M. M., Adams, V., Moore, R. J., Rood, J. I., & McClane, B. A. (2014). Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. *Future Microbiology*, *9*(3), 361–377. https://doi.org/10.2217/fmb.13.168

Vaishampayan, P., Yumoto, I., Yonghui Zeng, J., Strejcek, M., Smrhova, T., Junkova, P., & Uhlik, O. (2018). Whole-Cell MALDI-TOF MS Versus 16S rRNA Gene Analysis for Identification and Dereplication of Recurrent Bacterial Isolates. *Frontiers in Microbiology | Www.Frontiersin.Org*, *1*, 1294. https://doi.org/10.3389/fmicb.2018.01294

Walker, R. W., Clemente, J. C., Peter, I., & Loos, R. J. F. (2017). *The prenatal gut microbiome: are we colonized with bacteria in utero?* https://doi.org/10.1111/ijpo.12217

Wickramasinghe, S., Pacheco, A. R., Lemay, D. G., & Mills, D. A. (2015). Bifidobacteria grown on human milk oligosaccharides downregulate the expression of inflammation-related genes in Caco-2 cells. *BMC Microbiology*, *15*(1), 172. https://doi.org/10.1186/s12866-015-0508-3

Wong, C. B., Iwabuchi, N., & Xiao, J. (2019). Exploring the Science behind Bifidobacterium breve M-16V in Infant Health. *Nutrients*, *11*(8), 1724. https://doi.org/10.3390/nu11081724

Yang, J., & Yang, H. (2019). Antibacterial Activity of Bifidobacterium breve Against Clostridioides difficile. *Frontiers in Cellular and Infection Microbiology*, *9*. https://doi.org/10.3389/fcimb.2019.00288

Yun, B., Song, M., Park, D.-J., & Oh, S. (2017). Beneficial Effect of Bifidobacterium longum ATCC 15707 on Survival Rate of Clostridium difficile Infection in Mice. *Korean Journal for Food Science of Animal Resources*, *37*(3), 368–375. https://doi.org/10.5851/kosfa.2017.37.3.368