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Antibiotic screening of bifidobacteria and clostridia to improve selective media

Master´s thesis

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Sustainable Agriculture and Food Security

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Disclaimer

This master's thesis is submitted as a requirement for the Master´s degree in Sustainable Agriculture and Food Security at the Czech University of Life Sciences Prague and the Master´s degree in Biosafety and Food Quality at the University of Pisa. The submission is made concurrently to both universities under the double degree program agreement.While the substantive content of this thesis remains consistent for both submissions, there are variations in the title page and the roles of the supervisor and co-supervisor, adhering to the respective guidelines of each university.The dual submission of this thesis is a reflection of the academic collaboration and educational innovation fostered by the double degree program, aiming to broaden the academic and cultural perspectives of its participants.

Declaration

I hereby declare that I have authored this master's thesis carrying the name **Antibiotic screening of bifidobacteria and clostridia to improve selective media** independently under the guidance of my supervisor. Furthermore, I confirm that I have used only professional literature and other information sources that have been indicated in the thesis and listed in the bibliography at the end of the thesis. As the author of the master's thesis, I futher state that I have not infringed the copyrights of third parties in connection with its creation.

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1. Introduction

1.1 Bifidobacteria

The genus *Bifidobacterium* (*B.*) belongs to the phylum Actinobacteria, class Actinobacteria, subclass Actinobacteridae, order Bifidobacteriales, family *Bifidobacteriaceae*. Bifidobacteria were first isolated by Tissier in 1899 and were named *Bacillus bifidus*.

Bifidobacteria are y-shaped bacteria found in the intestines of mammals, and they're incredibly important for health (Hidalgo-Cantabrana, 2017).

Researchers have discovered nearly 90 species of these beneficial bacteria, each of them is thought to have different functions and health benefits (Chen, 2021). Currently, more than 100 bifidobacterial species are described (bacterio.net).

Despite their huge importance for the host, bifidobacteria typically make up less than 10% of the bacteria in the adult gut microbiome (Odamaki, 2016).

Because these bacteria positively contribute to the host health, they're often used as probiotics in supplements or particular foods. Probiotics are live microorganisms that provide a specific health benefit when consumed.

1.1.1 General description (morphology, physiology, metabolism)

Bifidobacteria are pleomorphic, asporigenic, obligate anaerobic or sometimes aerotolerant, chemoorganotrophic, immobile Gram-positive bacilli (Chen, 2021).

Bifidobacteria are currently acknowledged as being among the most significant bacteria used as probiotics to improve human health. Research has been conducted to find all-tolerant species to oxygen that would be easier to cultivate for use as probiotics, as their commercial application has been limited by their anaerobic nature (Li, 2010). *Bifidobacterium animalis subsp. lactis*, *Bifidobacterium asteroides* and other members of the family can grow under aerobic conditions (Lugli, 2017).

Bifidobacteria bacilli grow singly in chains or aggregates and their length varies on average from 2 to 5 μm. Bifidobacteria produce acid, but not gas from a wide variety of

carbohydrate substrates. They are catalase-negative with some exceptions (Chen, 2021).

The optimal growth temperature ranges from 37° to 41°C. No growth occurs below 20°C and above 46°C. The optimal pH of growth is between 6.5 and 7.0. No growth occurs at pHs below 4.5 and above 8.5. Bifidobacteria are acid-tolerant but not acidophilic microorganisms (Chen, 2021).

At the microscope, indeed, bifidobacteria appear as straight, bifurcated or highly branched rods. Moreover, when cultivated on nutritionally sub-optimal growth media, structural abnormalities, like bulbous swelling areas, frequently develop (Poupard et al., 1973). Bifidobacterial rods, either regular or irregular, may occur as individual cells or clusters of auto-aggregating cells (Rahman, 2008; Vlková, 2008).

Figure 1 (www.microbiologiaitalia.it) shows how bifidobacteria appear at the electronic microscope.

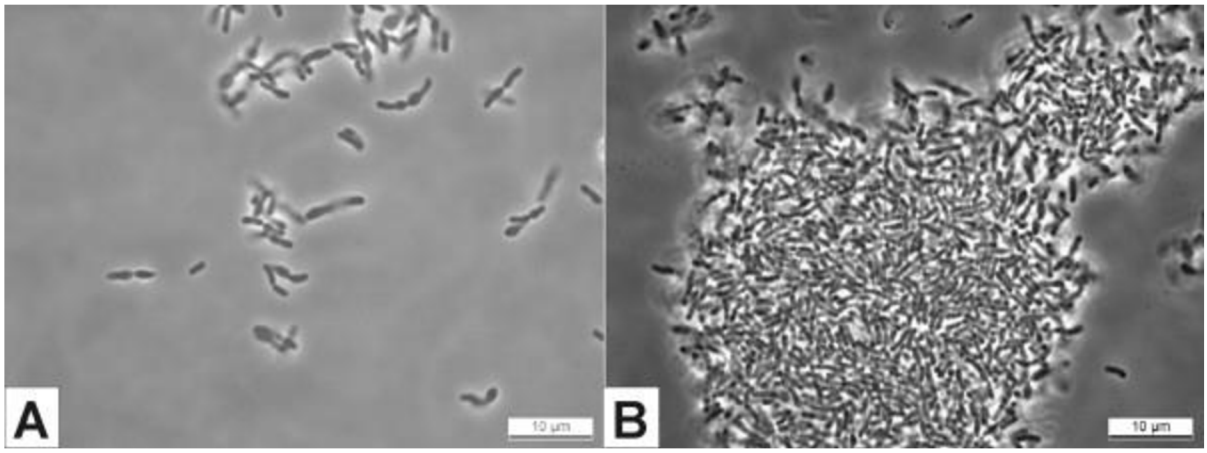

Figure 1 - Bifidobacteria appear at the electronic microscope. (www.microbiologiaitalia.it)

They can be found in blood and wastewater, likely as a result of environmental contamination, as well as the gastrointestinal tracts of humans and animals, the oral cavity, and the intestines of insects. Numerous bifidobacteria are praised for their purported health-promoting properties, as well as for their importance in colonization during the fetal period and for their role in infantile intestinal glycobioma (Lugli, 2017).

The definition given by the Italian Ministry of Health of antibiotic resistance is: "a natural biological phenomenon of adaptation of some microorganisms, which acquire the ability

to survive or grow in the presence of a concentration of an antibacterial agent, which is generally sufficient to inhibit or kill microorganisms of the same species" and is mainly due to incorrect or excessive use of antibiotics (www.salute.gov.it).

Antibiotic resistance in bifidobacteria is an increasing issue for both public health and the global economy. Current industrial dairy product strains of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* are frequently gentamicin, kanamycin, chloramphenicol, and tetracycline resistant. Despite the considerable work rising industries have put into process control and safety screening of commercial cultures, transmissible resistances nevertheless exist in industrial cultures (Nunziata, 2022).

A study by Ammor (2008) was aimed at studying the antibiotic resistance in 143 strains of lactic acid bacteria and bifidobacterial. The authors employed Etest and determined the MICs of six distinct antibiotics (chloramphenicol, clindamycin, erythromycin, streptomycin, tetracycline, and vancomycin). Most of the isolates were either sensitive to these antibiotics or were resistant to them based on the distribution of these MIC values. However, the bimodal distribution of the MIC range for some of these antibiotics suggests that some of the tested bacteria have acquired antibiotic resistance (Ammor, 2008).

1.1.2 Bifidobacterium spp. occurrence

Bifidobacteria are found naturally in the gastrointestinal tract of mammals but also in insects and birds. There are many species of bifidobacteria and it has been noted that different species can be found in different habitats, so it is not possible to say that the species are host specific. For example, species that were thought to colonize only the insect gut have been found in various hosts, including mammals (*B. actinocoloniiforme, B. asteroides, B. bohemicum, B. bombi,* and *B. indicum*) (Duranti, 2020).

In addition, it has been noted that the bifidobacteria present in the gastrointestinal tract of infants, where they are the first and major colonizers, often correspond to the same species present in the mother; thus, vertical transmission from mother to infant is hypothesized. In particular, strains of B. breve and B. longum have been isolated in infants (Duranti, 2020).

Figure 2 (Lugli, 2017) shows the taxonomy of bifidobacterial species.

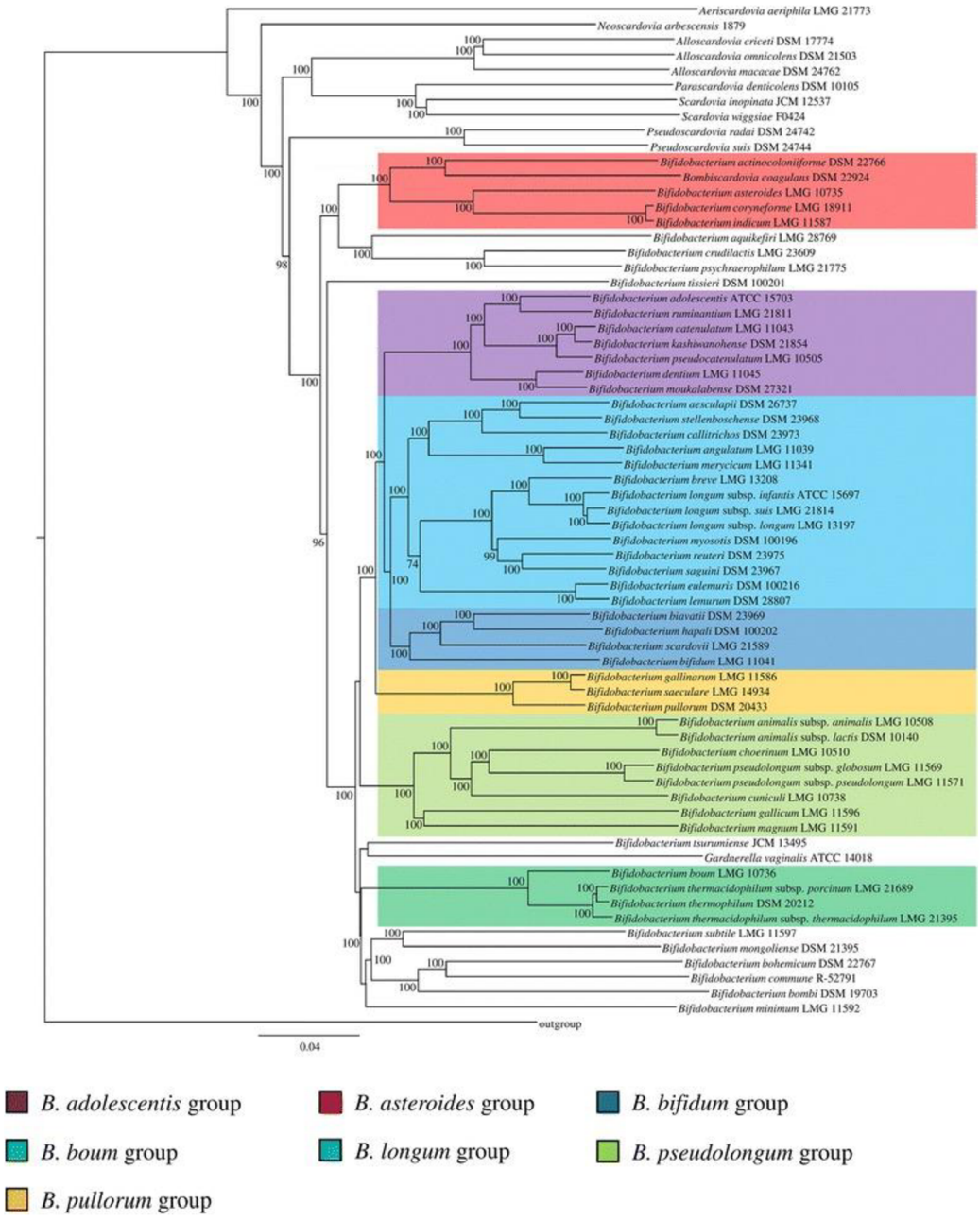

Figure 2 - Taxonomy of bifidobacterial species. (Lugli, 2017)

Although two specie of *Bifidobacterium* spp., *B. dentium* and *B. longum*, have been demonstrated to be involved in the aetiology of dental caries, none of them are currently thought to be of concern for human health (Brook & Frazier, 1993; Nakajo et al., 2010). On the other hand, it has been widely established that they are essential for preserving and reestablishing the healthy state of intestinal eubiosis. This is the rationale behind the widespread use of numerous *Bifidobacterium* strains as probiotics in a variety of foods, including infant formula, dairy products (primarily fermented milk and yogurts), and dietary supplements (Hidalgo-Cantabrana et al., 2017; Gomes & Malcata,

1999). Since early childhood, bifidobacteria have been known to have positive effects on their human hosts' health.

The presence of different species of bifidobacteria changes with age, from childhood to old age. *Bifidobacterium longum*, *B. breve*, and *B. bifidum* are generally dominant in infants, while *B. catenulatum*, *B. adolescentis*, and *B. longum* are more prevalent in adults (Aroboleya, 2016).

1.1.3 Role in gut microbiota

Bifidobacteria are found naturally in several ecological niches directly or indirectly linked to the gastrointestinal tract of animals, such as the human gut and oral cavity, insect intestines and wastewater (O'Callaghan, 2016).

The presence of bifidobacteria in the human gastrointestinal tract is associated with various health benefits such as, for example, development of the immune system or protection from pathogens. Furthermore, the metabolism of bifidobacteria is associated with the production of vitamin B9. For this reason, bifidobacteria are used as active ingredients in the diet, i.e., as probiotics (Duranti, 2020).

Due to their numerical advantage in breastfed infants compared to artificially lactated infants, bifidobacteria were quickly linked to a healthy intestinal tract; in fact, bifidobacteria and lactobacilli have been identified in breast milk (Cronin, 2011; Egan, 2021).

Furthermore, it has been demonstrated that some species of bifidobacteria, such as *B. animalis* ssp. *lactis*, have beneficial effects in the case of infectious diarrhea caused by viruses or bacteria (Duranti, 2020; O'Callaghan, 2016).

One of the main functions of this type of bacteria in humans is to digest fiber and other complex carbohydrates body can’t digest on its own. Fiber has been shown to help reduce weight gain and the risk of diabetes, heart disease and other chronic disorders. Bifidobacteria may help reduce the risk of these diseases by digesting fiber (Slavin, 2013).

That’s because when they digest fiber, these beneficial bacteria produce important chemicals called short-chain fatty acids (SCFAs). These compounds play several important roles for gut health and may also help control hunger (Ríos-Covián, 2016).

Bifidobacteria help produce other important chemicals too, including B vitamins and healthy fatty acids (Ríos-Covián, 2016).

They may also help prevent infections from other bacteria such as *Escherichia coli*, in part by producing chemicals that prevent toxins from passing into the blood (Fukuda, 2011).

Although it was once thought that the development of the intestinal microbiota took place at birth, the discovery of microbes in the placenta or amniotic fluid points to a primary fetal colonization. Additionally, the process is influenced by a variety of variables, all of which unquestionably have an impact on microbiota homeostasis. Early childhood is characterized by low diversity and complexity, with the main phyla being Actinobacteria, Proteobacteria, and Firmicutes. The composition of the gut microbiota undergoes significant changes during the first year of life before stabilizing around age 1-2. It is thought that after the third year of life, the gut microbiota develops an adult-like structure (Arboleya, 2016). In fact, populations of bifidobacteria are the most prevalent genus found in the healthy intestines of newborns after the optional anaerobes have used up all the oxygen. Levels significantly decline but remain stable during adulthood, and they decline once more as people age (Arboleya, 2016).

Furthermore, it has been established that bifidobacteria's capacity for environmental adaptation varies depending on their species. The gastrointestinal tract (GIT) of particular animals (e.g., *Bifidobacterium cuniculi* for rabbits, *Bifidobacterium angulatum* for cows, and *Bifidobacterium gallinarum* for chickens) or the human gut (e.g., *B. breve* and *B. longum* species) appears to be the best fit for some bifidobacterial species, according to scientific studies conducted up until recently. The distribution of *Bifidobacterium* species, however, is not host-specific, according to recent ecological studies based on Internally Transcribed Spacer (ITS) profiling. For instance, it was discovered that the *B. breve* species, which had previously only been linked to the human gut, is also present in domesticated animals. A number of species that were previously believed to be highly specialized to colonize the insect gut, including *Bifidobacterium actinocoloniiforme, B. asteroides, Bifidobacterium bohemicum, B. bombi*, and *Bifidobacterium indicum*, were also revealed to be widely distributed among various mammalian hosts. Notably, the presence of various bifidobacterial species in

various ecological niches supports the theory that such apparent horizontal transmission events may have been aided by anthropogenic influences (Duranti, 2020).

1.1.4 Use as probiotics

The term “probiotic” was redefined by the Food and Agriculture Organization of the United Nations (FAO) in 2001 as: “live microorganisms which, when administered in adequate quantities, confer a health benefit on the host”.

Probiotics are a wide range of microorganisms capable of providing general benefits to human health (Hill, 2014). Most probiotics currently available are lactic acid bacteria (LAB), *Bifidobacterium* spp. and some yeasts such as *Saccharomyces boulardii*, which have a long history of safe use and are legally “generally recognized as safe” (GRAS) (Judkins, 2020). To use the term probiotic, it is necessary to have a product with live microorganisms inside with a certain total count of 1×10^9 colony forming units (CFU). In particular, some strains belonging to some species of bifidobacteria and lactobacilli are considered valid for being probiotics since they show health benefits; the presence in literature of many articles about strains belonging to these two genera confirms they are among the most employed in the probiotic sector (Hill, 2014).

Probiotics are used to protect the digestive system during pharmacological treatments (such as, for example, antibiotics) and in any case can help those suffering from gastrointestinal tract pathologies such as irritable bowel syndrome. Bifidocateria are used in fermented foods such as yogurt and in those rich in fiber, but they can also be purchased in the form of supplements. Taking probiotics has no contraindications, only abuse can lead to unwanted effects (www.humanitas.it).

Here are some benefits that probiotic bifidobacteria can provide (Chen, 2021; Tamime, 1999):

- Anti-infective activity: thanks to the ability to colonize in the gastrointestinal tract.
- Anti-tumor activity: Bifidobacteria can inhibit tumors in animal models.
- Anti-inflammatory activity: Bifidobacteria can be used to inhibit oral and enteric infections such as irritable bowel syndrome.

- Enhanced lactose digestion
- Increase in fecal bifidobacterial.
- Decrease in fecal enzyme activity

Clinical studies that demonstrate the health advantages bifidobacteria have on their host have led to their widespread use as probiotic ingredients in foods that promote good health, particularly fermented dairy (Cronin, 2011). The strains frequently found in dairy products or other products containing probiotics intestinal probiotics belong to the species *B. bifidum, B. longum, B. breve, and B. infantis, B. animalis, B.adolescentis* (Kawasaki, 2006; www.iso.org).

In fact, over the past 20 years, probiotics have become increasingly popular as functional ingredients in pharmaceutical, food, and animal feed products. Probiotics are used in a variety of dairy products, including sour/fermented milk, yogurt, cheese, butter/cream, ice cream, and infant formula, making the dairy industry among the food industries the largest user of them. These probiotics are either used as starter cultures alone or in combination with conventional starters, or they are added to dairy products after fermentation, where their presence confers many functional characteristics to the product in addition to many health-promoting properties (for example, improved aroma, taste, and textural characteristics) (Gao, 2021).

Figure 3 (Chen, 2021) shows different positive effects related to the employment of different bifidobacterial strains.

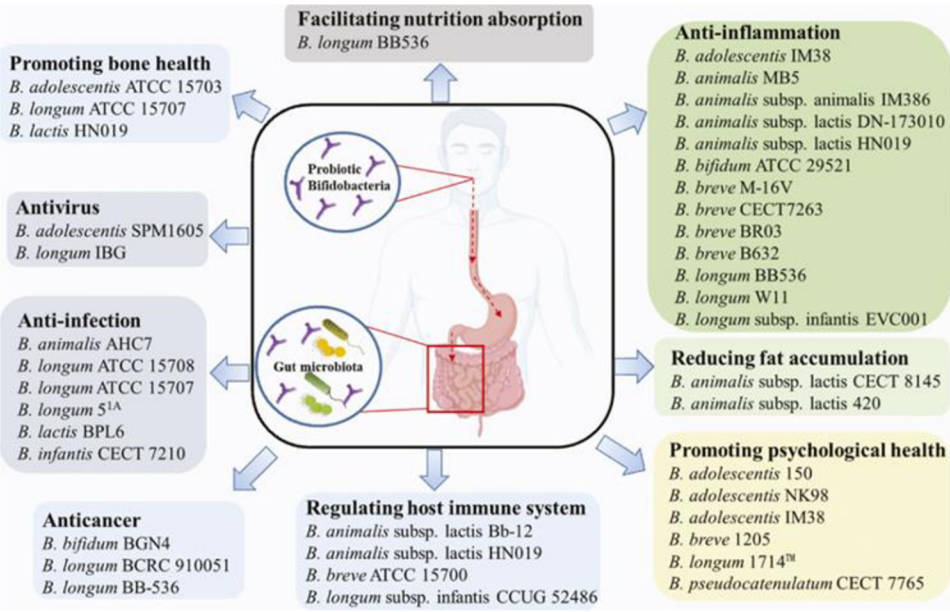

Figure 3 - - Different positive effects related to the employment of different bifidobacterial strains. (Chen, 2021)

1.1.5 Cultivation media and selective factors to detect bifidobacteria

Importantly, the bifidobacteria claimed to be present in dairy products survive in relatively large numbers of living cells (more than 10^6 per g) until consumption. Therefore, a rapid and reliable method for counting bifidobacteria is needed, both for routine determination of initial inoculum and for estimating the survival of bifidobacteria. Plate counting remains the preferred method for quality control measurements in dairy products. Therefore, selective media that promote the growth of bifidobacteria but suppress other bacteria are needed (Roy, 2003).

Media for bifidobacteria can be classified into basal media, differentiation media and selective media. Non-selective media such as RCM agar (reinforced clostridial medium) or MRS (de Man, Rogosa, Sharpe) are used for the detection of pure cultures in unfermented milk or fermented milk produced only with bifidobacterial, suitable for counting. Many selective or differential isolation media have been described for counting bifidobacteria in the presence of other lactic acid bacteria. There is no standard medium for the detection of bifidobacteria. However, for selective counting of bifidobacteria in food, Columbia agar medium supplemented with lithium chloride and

sodium propionate and MRS medium supplemented with neomycin, paromomycin, nalidixic acid and lithium chloride are recommended (Roy, 2003).

Focusing on the presence of bifidobacteria in dairy products, we can use ISO29981:2010 as a reference. In fact, ISO 29981 | IDF 220:2010 specifies a method for the selective enumeration of alleged bifidobacteria in dairy products using a colony counting technique at 37 °C under anaerobic conditions (www.iso.org).
The method is applicable to dairy products such as fermented and unfermented milk, milk powder, infant foods and starter culture in which these microorganisms are present and vital and in combination with other lactic acid bacteria (www.iso.org).

It was discovered in 2000 that 14 strains of lactic acid bacteria and 15 strains of bifidobacteria were resistant to the antibiotic mupirocin. Lactobacilli, lactococci, *Leuconostoc*, and streptococci growth in Wilkins-Chalgren broth was inhibited at mupirocin concentrations as low as 0.625–40 mg/l. On the other hand, mupirocin at a concentration of 300 mg/l had no effect on the growth of bifidobacteria in the same medium. The Wilkins-Chalgren agar, which contains mupirocin (100 mg/l), was subsequently developed as a selective medium for the isolation and counting of bifidobacteria in fermented dairy products. On this particular selective medium, the growth of lactobacilli, lactococci, *Leuconostocs*, and streptococci was completely inhibited. The recovery rates of bifidobacteria on this medium, however, were close to 100% leading to the conclusion that this medium is very selective and permits the growth of all common bifidobacteria strains. In probiotics with a mixed population of lactic acid and bifidobacteria, this medium can also be used for the selective counting of bifidobacteria. Furthermore, this medium also has the benefit of being simple to prepare (Koc, 2000).

A recent study aimed at obtaining a medium to allow the growth of bifidobacteria and inhibit the growth of clostridia (Vlková, 2015) proposed the employment of different selective factors for the detection of bifidobacteria. The medium in question was added with mupirocin, used as a selective factor together with glacial acetic acid. A

disadvantage of this medium, however, is that it does not have great selectivity when used on samples containing diverse microbiota.

1.2 Clostridia

Spore formers of the human intestinal microbiota include members of the family *Clostridiaceae*. This includes the genus *Clostridium* (*C.*), which is a strictly anaerobic sporogenic species. Although some species, such as *C. difficile* and *C. perfringens*, are known to be pathogenic, most *Clostridium* species observed in the gut have a commensal relationship with their hosts. In fact, it has been suggested that *Clostridium* groups IV and XIVa, *C. leptum,* and *C. coccoides* are involved in the prevention of inflammatory bowel disease (IBD) (Egan, 2021)

Bacteria of the genus *Clostridium* are found in soil, in the digestive tract of animals, and in water. In the gastrointestinal tract of animals, it is found in large numbers, especially in the large intestine, where it coexists with other microorganisms such as *Bacteroides, Enterococcus,* and *Lactobacillus* (Guo, 2020).

1.2.1 General description (morphology, physiology, metabolism)

Bacteria of the genus *Clostridium* are anaerobic, Gram-positive, sporogenic, rod-shaped and perform chemoorganotrophic metabolism. This means it can ferment a variety of nutrients, including polysaccharides, which are normally indigestible in the human gut (Guo, 2020). The genus *Clostridium* includes highly heterogeneous microorganisms with a wide range of phenotypes, including psycrhotrophic, thermophilic, and acidophilic (Lawson, 2016).

Observing clostridia under a microscope, they appear as elongated, irregularly shaped cells (often "drumstick" or "spindle" shaped) with a protuberance at one end (https://www.biomerieux.it/).

Figure 4 (Public Health Image Library – PHIL) shows an example of clostridium seen under the electronic microscope.

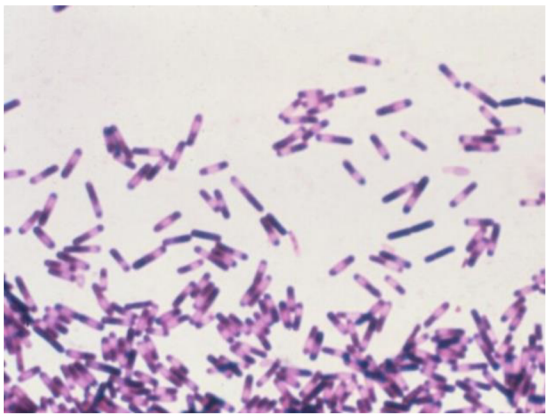

Figure 4 - clostridium seen under the electronic microscope. (Public Health Image Library – PHIL)

Clostridia physiology specializes mainly in acid production. Several pathways are known, including homoacetate fermentation by acetogens, propionate fermentation by *Clostridium propionicum*, and butyrate/butanol fermentation by the well-known solvent producer *C. acetobutylicum*. Clostridia degrade sugars, alcohols, amino acids, purines, pyrimidines, and polymers such as starch and cellulose (Durre, 2014).

The transmission of disease by pathogenic clostridia depends on their ability to form metabolically dormant, air-tolerant spores before leaving the host. The spores are highly resistant to extreme fluctuations in temperature and pressure, radiation, enzymatic digestion, and oxidizing agents, allowing them to survive for long periods of time and serve as an environmental reservoir for these organisms. Spores of *Clostridium perfringens*, *Clostridium botulinum*, *C. difficile* can be isolated from a variety of environments, including the gastrointestinal tract, animal carcasses, sewage, lawns, hospital rooms, and soil. Infections caused by these pathogens usually begin with ingestion of the spores, but *Clostridium perfringens* as well as *C. botulinum* can also enter the body through contaminated wounds (Shen, 2019).

The first morphological step in sporulation is the formation of polar septa, which produce two morphologically distinct but genetically identical cells. The large mother cell envelops the small forespore cell, leaving the forespore in the cytosol of the mother cell and surrounded by two membranes. A thick layer of modified peptidoglycan, known as the cortex, forms between the two membranes, giving the spores resistance to heat

and ethanol. A series of protein layers forms around the outer membrane of the forespore and protects it from enzymatic and oxidative attack. In *C. difficile*, an additional layer called exosporium forms, but this layer is not present in all sporogenic organisms (Shen, 2019).

In Figure 5, the sporulation process can be observed (Shen, 2019).

After detecting the germinating small molecules termed germinants, the spores of clostridial pathogens will germinate and grow into vegetative cells that secrete toxins (Shen, 2019).

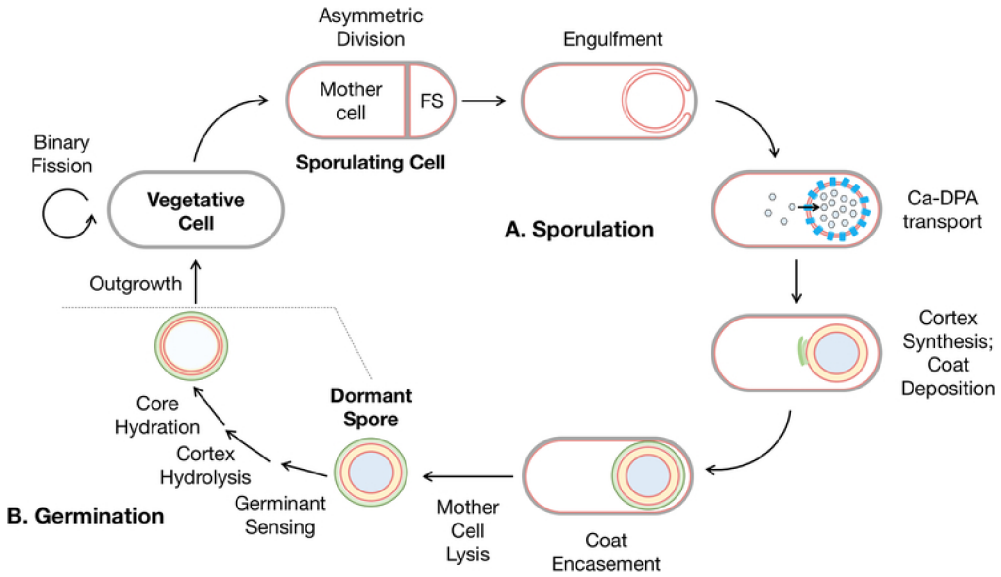

Figure 5. Life cycle of endospore formers. (Shen, 2019)

(A) Sporulation: after detecting certain environmental conditions, endospore formers activate Spo0A and initiate sporulation. The first morphological event is the formation of a polar septum, which creates a larger mother cell and a smaller forespore. The mother cell engulfs the forespore, and the two cells work together to assemble the dormant spore. Calcium dipicolinic acid (Ca-DPA) is synthesized in the mother cell and transported to the forespore in exchange for water. The cortex forms between the two membranes, and coating proteins polymerize on the surface of the membrane derived from the mother cell. Once the spore is mature, the mother cell lyses and releases the dormant spore into the environment. (B) Germination: after detecting the appropriate germinating small molecules, the spore initiates a cascade of signals that leads to activation of cortex hydrolases and hydration of the nucleus, which are necessary for metabolism to resume in the germinating spore.

Antibiotic resistance in clostridia is evolving into a major issue on a global scale. Studies that account for *Clostridium difficile*'s antibiotic resistance exist. Antibiotic resistance in C. difficile is concerning due to the treatment of infections caused by it as well as the possibility that the reservoir of AMR genes will spread to other pathogens (O'Grady,

2021). In addition, the occurrence of new *Clostridium difficile* strains is frequently accompanied by novel resistance. *C. difficile* has the highest level of antibiotic resistance at the moment. *C. difficile* exhibits multivariate antibiotic resistance. Changes in metabolic pathways and the creation of biofilms, as well as the acquisition of genetic elements and modifications of antibiotic target sites, all contribute to the survival of this pathogen in the presence of antibiotics (Spigaglia, 2018).

1.2.2 Clostridium spp. and close taxa occurrence

It is now generally accepted that the Clostridium I cluster represents the true genus *Clostridium* and that species within it should be recognized as *Clostridium sensu stricto*, although some species in the genera *Anaerobacter, Eubacterium*, and *Sarcina* have been found to be anomalous in the determination of *Clostridium* spp species belonging to the genus *Clostridium*. The classification of the genus *Clostridium* is continually evolving, with several important species involving rRNA cluster I (*Clostrioides difficile*) both internal (*Clostridium botulinum complex*) and external (*Cl. difficile*), which are medically important species. In particular, the *Clostridium botulinum* complex poses problems for the application of *16S rRNA* sequences, where four phylogenetically distinct clusters were identified predicted to represent different species or genera. This highlights the problem of describing taxa on the basis of individual traits, such as toxin production, which modern taxonomies can no longer support, especially when such traits are transferable, and a scientific contradiction will occur (Lawson, 2016).
Figure 6 (Lawson, 2016) shows the complete taxonomy of clostridia.

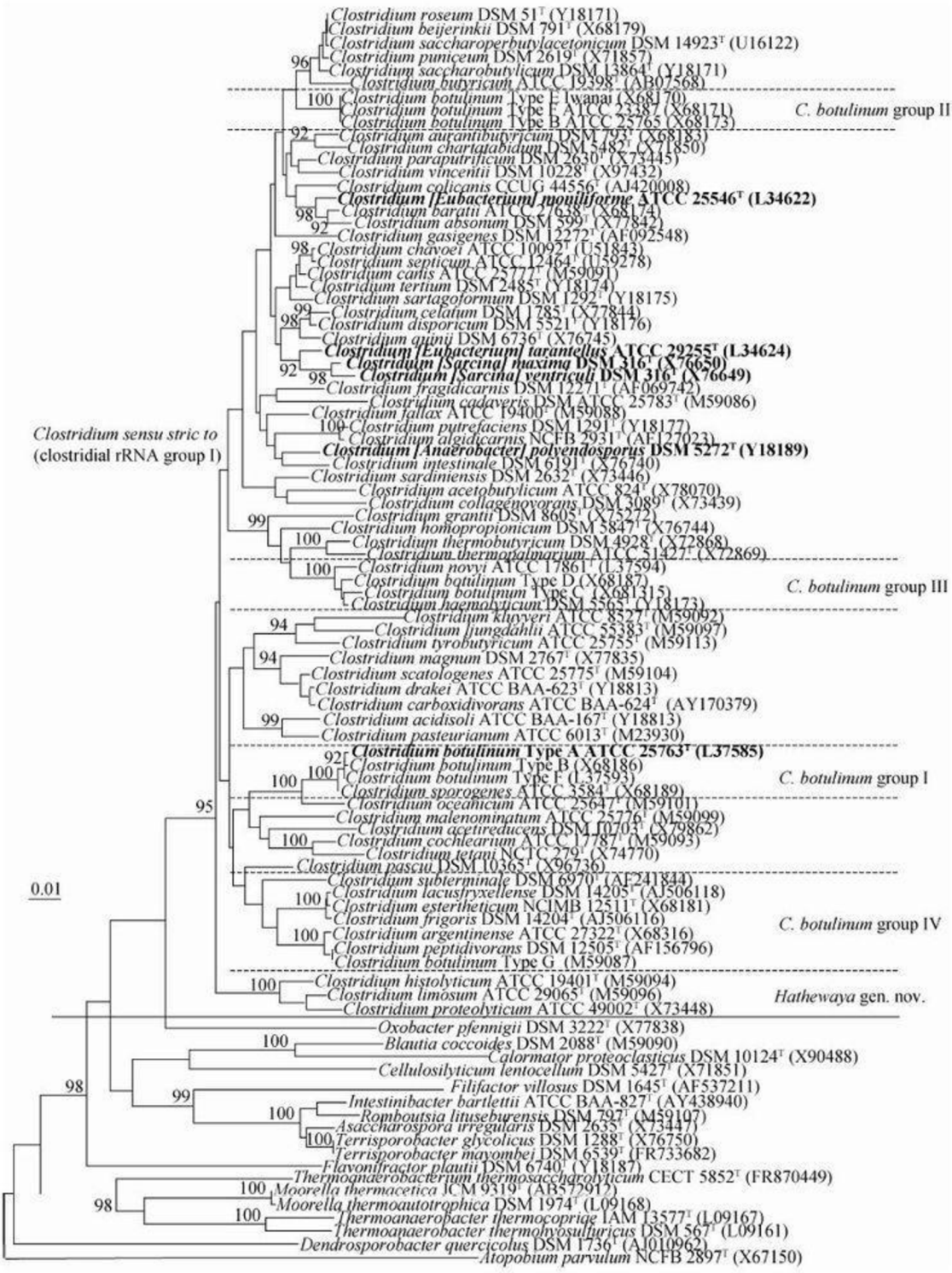

Figure 6 - Taxonomy of clostridia. (Lawson, 2016)

1.2.3 Role in gut microbiota

Although clostridia bacteria are primarily known as human pathogens, they can actually benefit humans through interactions with the gut. One example is the interaction with the immune system and the production of metabolites that benefit gut health (Guo, 2020). It is known that intestinal microorganisms interact closely with the epithelial immune system and trigger the differentiation and development of intestinal immune cells, maintaining an important symbiotic relationship between the host and microbes (Lawson, 2016). Another characteristic is the ability to generate SCFAs from carbohydrate fermentation with the help of other microbial species such as *Ruminaceae* and *Lachnospiraceae*. The fatty acids produced are used mainly by the liver, which regulates glucose and lipid metabolism (Guo, 2020). Specifically, SCFAs produced by sporogenic fiber-degrading bacteria are associated with improved intestinal homeostasis, suppression of inflammatory responses, and epithelial barrier function (Koopman, 2022).

1.2.4 Clostridial pathogenicity

Necrotizing clostridial infections of the skin and soft tissues have been described for centuries in the medical literature, and the main species involved, among others, are *Clostridium perfringens*, *Clostridium septicum, Clostridium sordellii, Clostridium histolyticum.* Infections caused by *C. perfringens* require extensive penetrating trauma, while *C. seticum* and *C. sordellii* can initiate infections spontaneously or in association with minor trauma or at birth. However, all these species can cause clostridia myonecrosis and gas gangrene. The epidemiology of these infectious diseases is therefore very diverse, but closely linked to human evolution. Spontaneous gas gangrene is caused by *Bacillus* sepsis and its higher incidence is associated with diseases such as neutropenia, gastrointestinal malignancies, radiation therapy, and chemotherapy. Recently, infections caused by *C. sordellii* and *C. novyi* have been reported to be associated with black tar heroin skin rash, normal pregnancy and delivery, and medication abortion (Stevens, 2012).

In particular, the toxicity of *Clostridium perfringens* is largely due to its reservoir of about 20 potent toxins (neurotoxic enterotoxins) that cause necrotizing enterocolitis, gangrene, and even death. *Cl. difficile* damages the colon through two toxins it produces, both enterotoxins (Guo, 2020; Stevens, 2012). Clostridia are the major producers of toxins among all bacterial species and are implicated in serious diseases in humans and other animals. The high efficacy of clostridial toxins is due to their specific targets with essential cellular functions and the types of changes they induce. Clostridial neurotoxins thus proteolyze key components of neural exocytosis. Botulinum neurotoxin inhibits neurotransmission at the neuromuscular junction, while tetanus toxin attacks inhibitory interneurons in the central nervous system (Popoff, 2009).

1.2.5 Cultivation media and selective factors to detect clostridia

Based on the *Clostridium* species used in different experiments, media for different *Clostridium* species have been evaluated in the literature. The suitability of a medium for a particular microorganism is based on both the selectivity of the antibiotic used and the ability of the agar to support the growth of the selected microorganism, which depends on the composition of the medium (De Jong, 2003). Because they are a fairly heterogeneous group, there is no available medium that allows all clostridia to grow while simultaneously excluding the growth of all competitive microbiota. Most clostridia reduce sulfite to sulfide, producing a fairly large black halo of iron sulfide in iron-containing media under anaerobic conditions. Therefore, blackening due to sulfite reduction serves as a diagnostic test for *Clostridium* spp. since most isolation media contain sulfite and appropriate iron salts. In addition to the diversity of metabolic activities and nutritional requirements, it is difficult to count both vegetative cells and clostridia spores using a single method. Many spores germinate only after heat activation, a process that kills the vegetative cells in the sample. Medium suitability therefore depends on the purpose of the study, the competitive flora within the sample, and the *Clostridium* species being studied (Bredius, 2003).

Over the years, numerous methods have been developed to isolate *Clostridium perfringens* from food, some of which have been incorporated into national and

international standards. A review of the literature shows that many different types of media and methods are used for the isolation of *Clostridium perfringens*, but there are a few more commonly used media. Tryptose-sulfite-cycloserine (TSC) agar is the most used one, followed by polymyxin-sulfite-sulfadiazine (SPS) agar, Shahidi-Ferguson-Perfringens (SFP) agar, and triptone sulfite-neomycin (TSN) agar. Most of these media have a similar base (peptone, yeast extract, sodium sulfite, ferrous ammonium citrate), but differ in the type of antibiotic used. Other media such as cycloserine azide sulfite agar (SCA), oleandomycin polymyxin sulfadiazine perfringens agar (OPSP) and differential clostridial agar (DCA) include meat extract, liver extract, TRIS buffer, glucose, or other ingredients such as starch are added (De Jong, 2003).

Clostridium butyricum isolation medium (BIM), a selective medium for the isolation of *C. butyricum* from human feces, is described. BIM is a synthetic minimal medium that contains trimethoprim, D-cycloserine, and polymyxin B sulfate as selective inhibitors. Qualitative tests have shown that *C. butyricum* and other butyrate-producing clostridia grow on BIM. BIM is useful for rapid isolation of *C. butyricum* from stool samples and should be useful in bacteriological studies of neonatal necrotizing enterocolitis (Popoff, 1984).

Regarding *Cl. difficile*, there are studies on selective and nonselective enrichment media. The efficiency of enriched media was tested based on the frequency of recovery of *Cl. difficile* from beef samples inoculated with *Cl. difficile*. In this study, brain heart infusion (TBHI) and cooked meat medium (TCM) containing sodium taurocholate were used as non-selective enrichment media for *Cl. difficile*, TCFB and TCDMN were used as selective media. Considering the analytical results, it was possible to conclude that TCM is the preferred enrichment medium for the recovery of *Cl. difficile* from beef samples, although the growth of *Cl. difficile* in TCFB and TCDMN did not provide satisfactory results (Chai, 2015).

1.3 Bifidobacteria and clostridia interactions

First and foremost, it's crucial to discuss the origins of bifidobacteria and clostridium in humans. The first colonization happens at birth; in fact, bifidobacteria are physiologically abundant only in young children, especially in their first year of life, and should be present, but this does not always happen, in pregnant women, from whom they would be inherited during childbirth and lactation; however, this does not always happen. They appear to make children more prone to atopia and obesity when they are absent (www.synalab.it). When given in the early years of life, antibiotics kill bifidobacteria, which is why they are absent in kids who were born vaginally, weren't breastfed, or whose mothers didn't have them. A physiological minimum of bifidobacteria in the adult, typically not more than 0.4%, is unlikely to be sufficient to ensure transfer to the unborn child. The data from the study under consideration show the exact opposite. In fact, bifidobacteria were found to increase by 10 times in the third trimester of pregnancy compared to the beginning of gestation, ensuring that the percentage of bifidabacteria is sufficient to make a mother-child transfer (Nuriel-Ohayon, 2019). Progesterone is the hormone that causes this increase in bifidobacteria in pregnant women, which raises the likelihood that they will transfer during delivery and lactation afterward. However, natural birth is essential for the proper transmission of bifidobacteria. If not sufficiently supplemented, babies born via caesarean section and those who received intrapartum antibiotic therapies lack or are severely deficient in bifidobacteria (Korpela, 2018). A first possible transmission of bifids to the newborn is guaranteed by natural delivery because of the likelihood of bifidobacteria in the vagina and the possibility of maternal feces contaminating the baby.

Another sign of the transmission of bifobacteria from the mother to the child is the newborn's natural breastfeeding.

Infants were the main hosts for bifidobacteria of the species *B. bifidum, B. short, and B. longum* (Duranti, 2019).

The statistical analysis of the study in question (Ferraris, 2012) revealed that the neonatal intensive care unit was a deciding factor in influencing the colonization of post-natal by clostridia and that antibiotic cycles affected colonization levels with regard to

clostridia, the presence of which was found primarily in premature infants (PN). Approximately, 80% of PNs have been colonized, many of them after the seventh week of hospitalization, according to this study, which documents a high average incidence of clostridial colonization during hospitalization. The three species that were isolated the most frequently were *C. difficile, C. butyricum, and C. perfringens.* Neonatal intensive care units frequently use broad-spectrum antibiotics not considering the risk related to PN infections, which raises the risk of colonization with resistant strains. The incidence of clostridial colonization increasing during hospitalization raises the possibility that environmental factors play a role in colonization. Indeed, clostridia are sporogenic bacteria that can survive in hospital environments despite being strictly anaerobic (Ferraris, 2012).

We can say that *C. perfringens* is connected to the potentially fatal condition necrotizing enterocolitis (NEC) in premature infants. According to the study, *C. perfringens* carriers of the toxin gene colonize about one-third of premature babies 3 weeks after birth. However, research has shown that using more breast milk, oxygen, and antibiotics causes the intestine to become hostile to the growth of C*. perfringens*. No specific toxin was connected to NEC, despite the prevalence of potentially toxic *C. perfringens* isolates (Shaw, 2020).

There are several research showing beneficial interactions regarding bifidobacteria and clostridia. For example, one research shows that long-term chronic unpredictable mild stress (CUMS) exposure can lead to redox imbalance and inflammation, which can compromise the integrity of the intestinal barrier. This study investigated the effects of a combination of probiotic bacteria, including *C. butyricum* and *B. infantis*, on rats exposed to multiple low-intensity stressors for 28 days. Probiotic supplementation significantly reduced CUMS-induced inflammation and further altered T lymphocyte levels. Histological changes and improvements in intestinal barrier integrity confirm the beneficial effects of probiotic blends on changes in intestinal morphology caused by CUMS. In summary, the results presented in the study suggest that the combination of *C. butyricum* and *B. infantis* significantly reduces oxidative stress and inflammation (Fatima, 2023).

Another study has shown that antibiotic-associated diarrhea (AAD) is one of the most common complications of most types of antibiotics. Our objective was to determine the efficacy of *C. butyricum*, *B. infantis* and their mixture in treating AAD in mice. Single probiotic strains and probiotic mixtures were administered to AAD models for short- and long-term periods to evaluate changes in gut microbiota composition and diversity, colonic histopathology, and systemic inflammation. Data obtained showed that long-term rather than short-term probiotic therapy had a positive effect on restoring the gut microbiota, restoring tissue structure, and reducing systemic inflammation. Short-term administration of probiotic strains or mixtures showed no obvious beneficial effects on AAD. Furthermore, the beneficial effects of *C. butyricum* in combination with probiotic mixtures of *B. infantis* were greater than those of their individual strains. In this study, supplementation with *C. butyricum* in combination with a probiotic mixture of *C. butyricum* and *C. Infantis* may be a simple and effective method to treat AAD (Ling, 2015).

2. Aim of the thesis

Bifidobacteria are commensal bacteria found mainly in the digestive tract of humans and animals, helping maintain the balance of the intestinal microbiota and thus favorably influencing the health of their host. In contrast, clostridia include common commensals as well as highly pathogenic representatives of this taxon. Both groups of microorganisms are resistant to mupirocin, and sensitivity to norfloxacin is quite variable, so it is impossible to quantify them separately by cultivation on available selective media.

The work was aim to write a literature review regarding the mentioned groups of microorganisms and the given issue. In the experimental part, different species of bifidobacteria and clostridia were tested against selected antibiotics to find suitable selective factors for their culture determination.

In particular, strains tested for their sensitivity to a selected set of antibiotics using the disk diffusion method. This screening enabled the selection of antibiotics for testing by the e-test method.

The diffusion and dilution method were used to determine the sensitivity of selected bacteria to acetate, lactate, formate, and propionate.

Based on the obtained results, modifications of the existing selective media will be proposed.

3. Material and Methods

3.1 Strains collection

3.1.1 Bifidobacteria

In total, 38 bifidobacterial strains were selected for testing. These strains belong to different *Bifidobacterium* spp. as is shown in Table 1.

No.	Code	*Species*	Origin
1	B001	*Bifidobacterium breve*	stool of infant
2	B002	*Bifidobacterium animalis* subsp. *lactis*	stool of infant
3	B003	*Bifidobacterium longum*	stool of infant
4	B004	*Bifidobacterium bifidum*	stool of infant
5	B005	*Bifidobacterium animalis* subsp. *lactis*	yoghurt
6	B006	*Bifidobacterium dentium*	dental caries
7	B007	*Bifidobacterium longum* subsp. *infantis*	(stool of infant)
8	B008	*Bifidobacterium adolescentis*	intestine of adult
9	B009	*Bifidobacterium bifidum*	intestine of adult
10	B010	*Bifidobacterium animalis* subsp. *animalis*	rat faeces
11	B011	*Bifidobacterium catenulatum/pseudocatenulatum*	stool of adult
12	B012	*Bifidobacterium adolescentis*	stool of adult
13	B013	*Bifidobacterium animalis* subsp. *lactis*	stool of adult
14	B014	*Bifidobacterium pseudocatenulatum*	stool of infant
15	B015	*Bifidobacterium catenulatum* subsp. *kashiwanohense*	stool of infant
16	B016	*Bifidobacterium bifidum*	stool of breast-fed infant
17	B017	*Bifidobacterium catenulatum*	stool of infant
18	B018	*Bifidobacterium dentium*	stool of infant
19	B019	*Bifidobacterium breve*	stool of infant
20	B020	*Bifidobacterium breve*	stool of infant

21	B021	*Bifidobacterium ruminantium*	GLT monkey faeces
22	B022	*Bifidobacterium adolescentis*	GLT monkey faeces
23	B023	*Bifidobacterium adolescentis*	GLT monkey faeces
24	B024	*Bifidobacterium adolescentis*	GLT monkey faeces
25	B025	*Bifidobacterium adolescentis*	GLT monkey faeces
26	B026	*Bifidobacterium adolescentis*	GLT monkey faeces
27	B027	*Bifidobacterium faecale*	GLT monkey faeces
28	B028	*Bifidobacterium faecale*	GLT monkey faeces
29	B030	*Bifidobacterium breve*	infant faeces
30	B031	*Bifidobacterium breve*	infant faeces
31	B032	*Bifidobacterium dentium*	infant faeces
32	B033	*Bifidobacterium catenulatum*	infant faeces
33	B034	*Bifidobacterium dentium*	monkey faeces
34	B035	*Bifidobacterium pseudolongum subsp. pseudolongum*	monkey faeces
35	B036	*Bifidobacterium pseudolongum subsp. globosum*	monkey faeces
36	B037	*Bifidobacterium olomucense VB26T*	monkey faeces
37	B038	*Bifidobacterium moraviense VB25T*	monkey faeces
38	B039	*Bifidobacterium sp. nov. VB17*	monkey faeces

Table 1 – Bifidobateria strains

3.1.2 Clostridia

In total, 29 strains were selected for testing. These strains belonged to different *Clostridium* spp., and other clostridia taxa as is shown in Table 2.

No.	**Code**	***Species***	**Origin**
1	C001	*Paeniclostridium sordelli*	ant-eater
2	C002	*Paeniclostridium bifermentans*	ant-eater
3	C003	*Clostridium colicanis*	ant-eater
4	C004	*Clostridium perfringens*	ant-eater
5	C005	*Clostridium barati*	ant-eater
6	C006	*Clostridium perfringens*	infant
7	C007	*Clostridium perfringens*	boulette - Hamburger (type strain)
8	C008	*Clostridium neonatale*	infant
9	C009	*Clostridium perfringens*	infant
10	C010	*Clostridium symbiosum*	infant
11	C011	*Clostridium colicanis*	ant-eater
12	C012	*Clostridium perfringens*	ant-eater
13	C013	*Paeniclostridium bifermentans*	infant
14	C014	*Clostridium neonatale*	infant

15	C015	*Clostridium butyricum*	infant
16	C016	*Clostridium tertium*	infant
17	C017	*Paeniclostridium sordelli*	infant
18	C018	*Clostridium perfringens*	calf
19	C019	*Clostridium tertium*	no data (type strain)
20	C021	*Enterocloster clostridioformis*	adult
21	C022	*Clostridium tertium*	infant
22	C023	*Clostridium perfringens*	calf
23	C024	*Clostridium tertium*	no data
24	C025	*Clostridium butyricum*	infant
25	C027	*Clostridium perfringens*	fish
26	C028	*Clostridioides difficile*	no data
27	C030	*Clostridium perfringens*	dog
28	C032	*Clostridium butyricum*	calf
29	C034	*Clostridium paraputrificum*	human faeces

Table 2 – Clostridia strains

3.2 Selected antibiotic (ATB) for screening

3.2.1 Antibiotics for disk diffusion method

Antibiotic screening was performed on 65 different antibiotics (all Oxoid, UK), (Table 3). The selection of used antibiotics was done based on previous antibiotic sensitivity screenings (Vlkova, 2015; Makovska, 2021). Kirby-Bauer disk diffusion method provided for the evaluation of the diameters of the inhibition zones around the disks containing the antibiotic tested (Tascini, 2016). In figure 7 it is shown how this method works, especially the readout of the results (Sharma, 2022). Kirby-Bauer did not provide MIC (minimum inhibitory concentration) values, but rather the diameter of the zone of inhibition (Tascini, 2016).

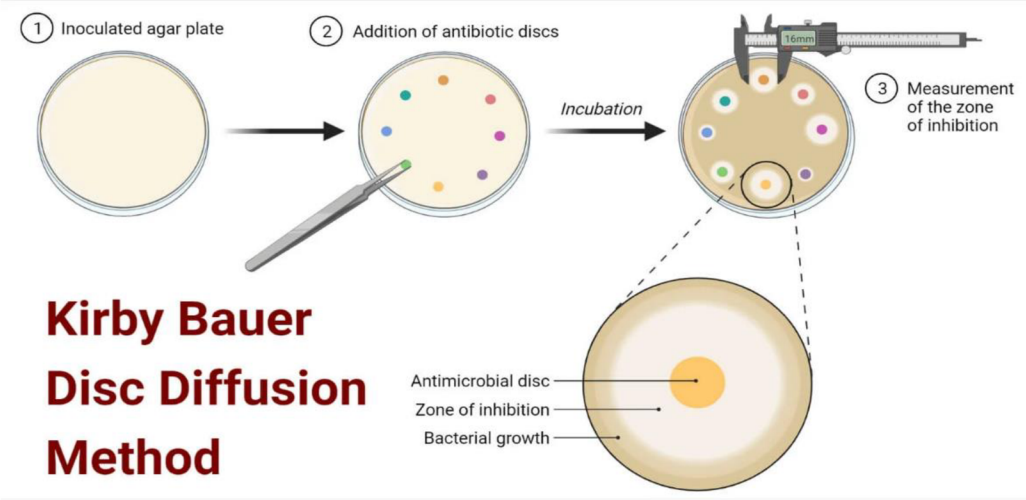

Figure 7 - Kirby Bauer Disc Diffusion Method for Antibiotic Susceptibility Testing. (Sharma, 2022)

In Table 3 ATB are classified in alphabetical order by the full name, the acronym and dose used and in which testing groups they were organized for cultivation on Petri dishes:

ANTIBIOTICS	ACRONYM	DOSE	TESTING GROUP
Amikacin	AK	30 µg	11
Amoxycilin	AML	25 µg	2
Amoxycilin + Clavulanic acid	AMC	30 µg	2
Ampicilin	AMP	2 µg	2
Ampicilin	AMP	10 µg	2
Ampicilin + Sulbactam	SAM	20 µg	2
Azitromycin	AZM	15 µg	6
Aztreonam	ATM	30 µg	2
Bacitracin	B	10 IU	7
Baytril R/Reg tm of Bayer	ENR	5 µg	10
Cefaclor	CEC	30 µg	3
Cefepime	FEP	30 µg	3
Cefoperazone	CFP	75 µg	4
Cefovecin	CVN	30 µg	4
Cefoxitin	FOX	30 µg	4
Ceftaroline	CPT	5 µg	4
Ceftazidime	CAZ	10 µg	4
Ceftibuten	CFT	30 µg	4
Ceftriaxone	CRO	30 µg	4
Cefuroxime	CXM	30 µg	5

Chloramphenicol	C	30 µg	7
Ciprofloxacin	CIP	5 µg	11
Clindamycin	DA	2 µg	10
Colistin	CT	10 µg	7
Doripenem	DOR	10 µg	3
Doxycycline	DO	30 µg	8
Ertapenem	ETP	10 µg	6
Erythromycin	E	15 µg	6
Fluconazole	FCA	25 µg	1
Flumequine	UB	30 µg	5
Fosfomycin	FOS	50 µg	11
Framycetin	FY	100 µg	11
Gentamicin	CN	10 µg	10
Gentamicin	CN	30 µg	10
Gentamicin	CN	120 µg	10
Kanamycin	K	30 µg	1
Lincomycin	MY	10 µg	6
Linezolid	LZD	10 µg	7
Lomefloxacin	LOM	10 µg	9
Mecillinam	MEL	10 µg	9
Meropenem	MEM	10 µg	6
Minocycline	MH	30 µg	8
Moxifloxacin	MXF	5 µg	11
Mupirocin	MUP	200 µg	1
Nafcillin	NF	1 µg	10
Nalidixic acid	NA	30 µg	5
Neomycin	N	10 µg	1
Netilmicin	NET	10 µg	1
Nitrofuratoin	F	100 µg	7
Norfloxacin	NOR	10 µg	5
Ofloxacin	OFX	5 µg	11
Oleandomycin	OL	15 µg	6
Oxolinic acid	OA	2 µg	5
Oxytetracycline	OT	30 µg	8
Pefloxacin	PEF	5 µg	9
Penicillin G	P	10 µg	9
Piperacillin	PRL	30 µg	3
Piperacillin/Tazobactam	TZP	36 µg	3
Polymyxin B	PB	300 IE	8
Quinupristin/Dalfopristin	QD	15 µg	9
Spiramycin	SP	100 µg	7
Streptomycin	S	300 µg	1
Sulphafurazole	SF	300 µg	9
Tetracycline	TE	30 µg	8
Ticarcillin	TIC	75 µg	3

Tobramycin	TOB	10 µg	1
Vancomycin	VA	5 µg	5

Table 3 – Antibiotics

3.2.2 Antibiotics for e-test

With the help of broth micro-dilution (BMD) and automated systems, it was possible to obtain minimum inhibitory concentrations (MICs), which is the lowest concentration of antibiotic that can inhibit the in vitro growth of microorganisms after 18-24 hours of incubation. The E-test is a manual method performed in agar using a paper strip with a concentration gradient of a specific antibiotic suitable for determining MIC. MIC value was determined at the intersection of the strip and the growth inhibition ellipse. Suitable as a supplement to other methods (Tascini, 2016; Balouiri, 2016). In Figure 8 (LeCorn, 2007) and 9 (Manno, 2003) the operation of the E-test is shown.

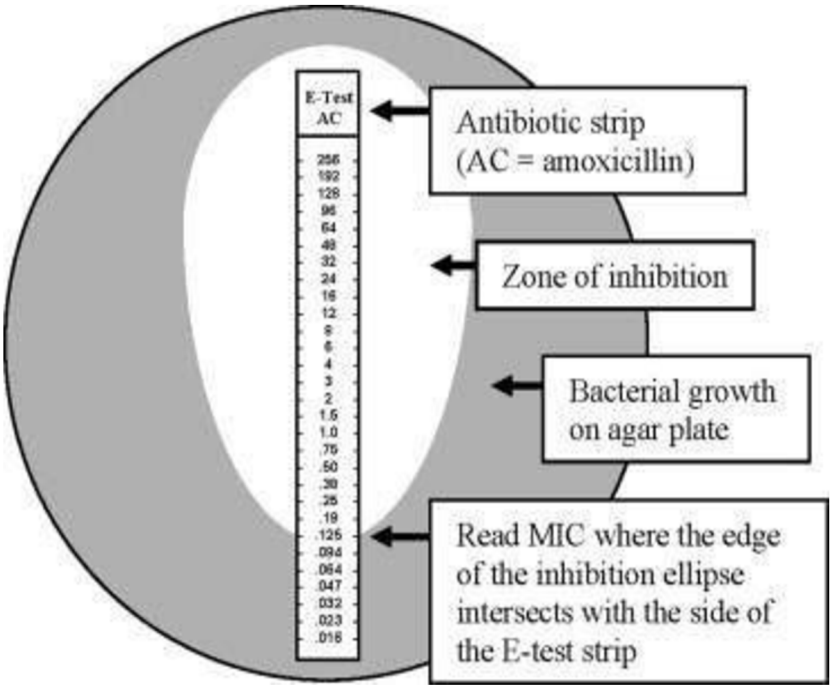

Figure 8 - (LeCorn, 2007)

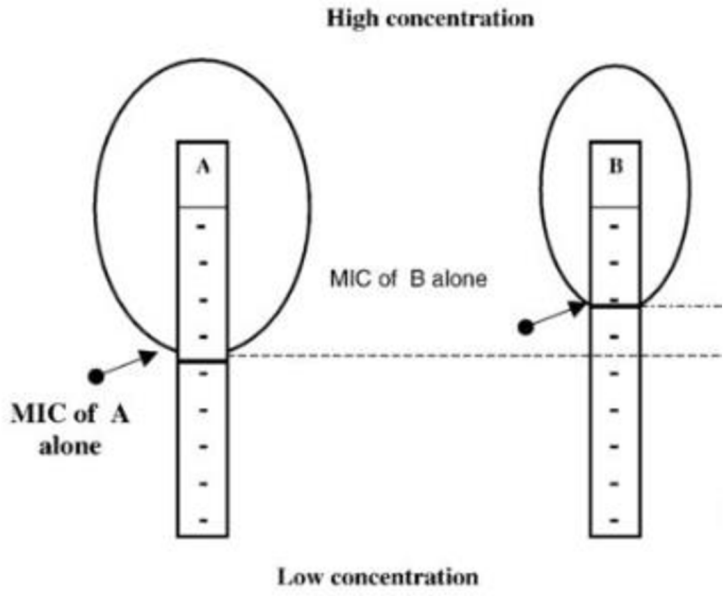

Figure 9 - Interpretation of the E test combination study, showing MICs of antibiotics A and B. (Manno, 2003)

The E-test was performed with 3 selected antibiotics, Nalidixic acid (NA), Ceftibuten (CFT) and Mecillinam (MEL) chosen according to the results obtained by Kirby-Bauer disk diffusion method.

3.2.3 Other tested antimicrobials

In addition to antibiotics, acids were also analyzed as antimicrobials. The acids used were lactic acid, acetic acid, propionic acid, and formic acid. The acids were employed at concentrations: 0.5%, 1%, and 2%.

3.3 Media preparation

The strains used were routinely cultivated in the broth media. The antibiotic screening was done with use of the same media, however, in the form of agar. The preparation is described below.

3.3.1 Broth media for tested strain cultivation

To inoculate the bifidobacteria and clostridia bacteria taken from the culture collection of the Department of Microbiology, Nutrition a Dieteticts, it was necessary to prepare tubes containing a modified medium named WSP. WSP medium was prepared using 1000 ml of water, 33 g of Wilkins Medium Broth, 0.5 mL of polysorbate, 1 g of cysteine and 5 g of soya peptone. Making a proportion it was possible to modify the quantities for the necessary dose. Once all the ingredients were placed in a flask, the mixture was boiled for 40 minutes. Subsequently, with the help of an electronic pipette, 9 mL of liquid medium were inserted into each tube. The tubes, still open, were placed in a hot bath at 85°C for 15 minutes. After the necessary time, using a complex system for anaerobiosis, anaerobiosis inside the tubes was creates and the tubes were closed with a specific stopper according to Hungate (Hungate, 1969; Hungate, 1973). Finally, the tubes were placed in an autoclave for 50 minutes. After sterilization and cooling down the tubes were ready to be used.

3.3.2 Agar media for antibiotic sensitivity screening

For plate cultivation of anaerobic bacteria, it was necessary to prepare another type of medium, Wilkins Chalgren Anaerobe Agar. An anaerobic environment is then created during cultivation using Atmosphere Generation System (ANAEROGENTM COMPACT, Oxoid). This medium was prepared using 1000 mL of water, 42.97 g of Wilkins Chalgren Agar, 1 mL of tween polysorbate, 0.5 g of cysteine and 5 g of soya pepton, in the right doses. Making a proportion it was possible to modify the quantities for the necessary dose. All the ingredients were placed in a flask and placed in an autoclave for 50 minutes. Once the sterilization was complete, the medium was left to cool down in a water bath at 52°C.

3.3.3 Other used media

Another medium that was used to make the dilution line for quantification of bacteria. The dilution line medium was used to test the resistance to acids at different

concentrations. The procedure was the same as the preparation of the tubes, but the ingredients were different: 1000 mL of water, 5 g of Nutrient broth, 2.5 g of Yeast ed, 5 g of Tryptone, 0.5 mL of tween polysorbate and 0.25 g of cysteine. Making a proportion it is possible to modify the quantities for the necessary dose. For the procedure refer to chapter 3.3.1 Media for cultivate bacteria (tubes).

3.4 Sensitivity screening

3.4.1 Disk diffusion method and e-test

The cultures of bifidobacteria and clostridia from the collection were inoculated in the tubes (0.3 mL of the frozen culture to 9 mL of media) and cultivated for 24 hours at 37°C. The tube with the grown microorganisms was analyzed through a microscope (phase contrast microscopy). It was observed that the culture was pure and not contaminated. If the culture was pure, then cultivation by inclusion was carried out. Since 11 groups of antibiotics and 4 acids were used, 13 petri dishes were required for each bacterial strain. 1 mL was taken from the tube containing the microorganisms with a syringe and placed on the petri dish. With a graduated pipette, 25 mL of modified Wilkins Chalgren Anaerobe Agar were taken and inserted into the Petri dishes. Eight movements homogenized the medium for the culture of microorganisms. Then the agar had to solidify.

A precaution to be taken for clostridia is the production of gas, therefore when they were taken from the tube one should be careful and eliminate the gas using the syringe. After the agar had solidified, the antibiotic plates could be inserted. Six antibiotic plates were inserted into each Petri dish using Antimicrobial Susceptibility disc Dispenser (Oxoid). Once the preparation of the plates with the antibiotics was complete, they were inserted into the jars for the anaerobic, the anaerobic environment was carried out (ANAEROGENTM COMPACT, Oxoid), and they were placed in an incubator at 37°C for 24 hours.

After 24 hours of cultivation in the incubator, the results were ready to read. The surrounding halo was visible (figure 10 and 11). The minimum value was 6 mm (the size of the antibiotic disc) or the size of the antibiotic plate. If the value was 6 mm, the

bacterial strain is resistant to used concentration of the antibiotic. While the larger the halo, the more sensitive the microorganism will be to the antibiotic or acid.

In 2019 EUCAST (European Committee for Antimicrobial Susceptibility Testing) changed the definition of the susceptibility test types S and R, as indicated below (www.infezioniobiettivozero.info):

S – Standard and Sensitive Dosing Regimen: an organism is classified as “S” when there is a high probability of successful treatment using a standard antibiotic regimen.

R - Resistant: an organism is classified “R” when there is a high probability of treatment failure even in the presence of increased exposure to antibiotics.

In Figure 10 and 11, it is possible to clearly see the presence and absence of halos and therefore the resistance (R) or sensitivity (S) of the microorganisms to antibiotics.

Figure 10

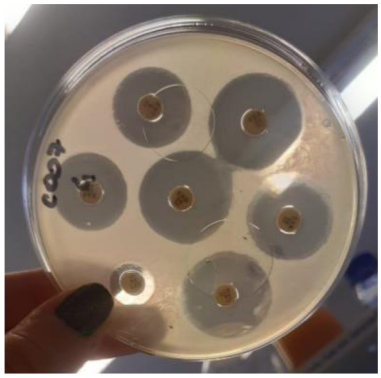

Figure 11

After analyzing all the species of bifidobacteria and clostridia selected, five species of bifidobacteria and five species of clostridia were chosen to proceed with the E-test (see Table 4).

Code	Species
B005	*Bifidobacterium animalis* subsp. *lactis*
B012	*Bifidobacterium adolescentis*
B015	*Bifidobacterium catenulatum* subsp. *kashiwanohense*
B019	*Bifidobacterium breve*
B039	*Bifidobacterium sp. nov. VB17*
C007	*Clostridium perfringens*

C008	*Clostridium neonatale*
C015	*Clostridium butyricum*
C017	*Paeniclostridium sordelli*
C028	*Clostridioides difficile*

Table 4

Preparation of Petri dishes for E-test is identical to preparation of antibiotic test plates. After culturing the bacterial strains, wait for the agar to solidify and place the antibiotic strip in the center of the Petri dish by applying slight pressure. It was placed in an anaerobic environment and incubated at 37°C for 24 hours.

Once the 24 hours passed, results were ready to read.

For each antibiotic strip it was possible to observe the exact degree of resistance or sensitivity based on the size of the halo. If the halo didn't develop, the microorganism was resistant to the antibiotic.

In photos 12,13 and 14 it is possible to see the halos around the antibiotic strips.

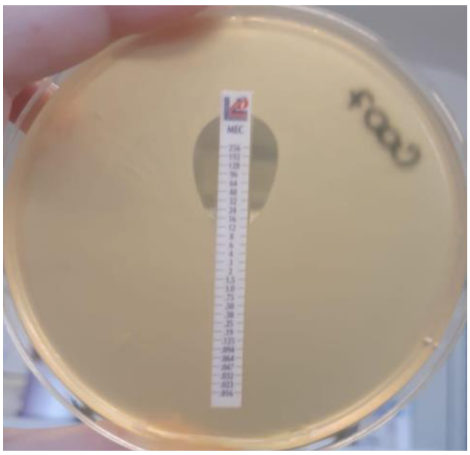

Figure 12

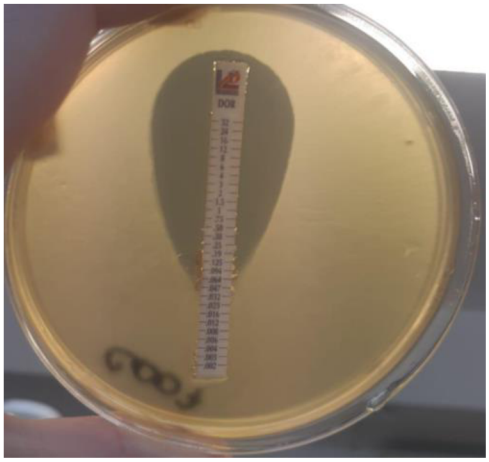

Figure 13

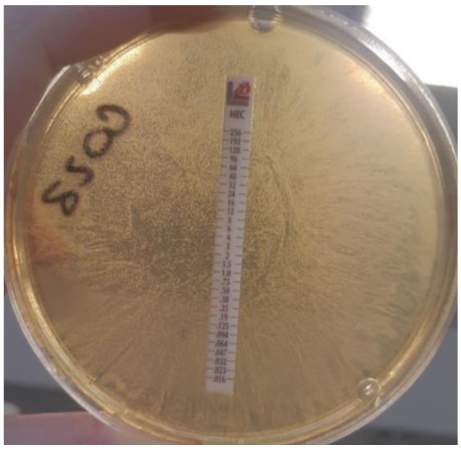

Figure 14

3.4.2 Agar diffusion method with acids

As for the acids, the desired concentration of acids was prepared. In this case four acids were employed: lactic acid, acetic acid, propionic acid, and formic acid. For each acid we work on 3 concentrations: 0.5%, 1% and 2%. For acetic acid, propionic acid, and formic acid, a was created starting from an almost pure initial solution of “100%” acid and a proportion was made to know the doses of water and acid to use to reach desired concentrations. While for lactic acid a 10% acid solution was needed, so the doses changed. Tables 5 and 6 show the proportions of acid and water used to arrive at the desired concentrations.

Acetic, Propionic and Formic AC		
%	Acids	Water
0,50%	10 µL	1990 µL
1%	20 µL	1980 µL
2%	40 µL	1960 µL

Table 5

Lactic AC		
%	Acids	Water
0,50%	100 µL	1900 µL
1%	200 µL	1800 µL
2%	400 µL	1600 µL

Table 6

To insert the different concentrations of acids in the petri dish holes were made inside the agar thanks to sterile applicator. Here 50 µL of acid were inserted. It was important to write the concentration and the name of the acid that was going to be inserted inside each single hole on the petri dish. Once the preparation of the plates with the acids was complete, they were inserted into the jars for the anaerobic, the anaerobic environment was carried out, and they were placed in an incubator at 37°C for 24 hours. After 24 hours results were ready.

After 24 hours in the incubator, the results were ready. The surrounding halo was visible. The minimum value was 6 mm or the size of the hole on the plate. If the value was 6 mm, the bacterial strain was resistant, while the larger the halo, the more sensitive the strain would be to the antibiotic or acid.

3.4.3 Cultivation and quantification of strains in media supplemented with acids

Since the bacteria were found to be too sensitive to acids, they were then inoculated with different acids (acetic, formic, lactic, and propionic) in different concentration (0.25, 0.5, and 1).

The first step was inoculating two bifidobacteria and three clostridia.

Each bacterium had one tube with the pure sample, three for each acid with different concentrations. 0,25% and 0.5% were used to make the dilution line, while 1% helped measure the optical density.

Then a test tube was made with 0.5 ml of sample and 0.5 of medium. While the ones with the acids were composed of 0.5 mL of the sample + 0.5 mL of the acid dilution.

Before incubating the tubed optical density was measured, then they were incubated at 37°C for 24/48 hours and the optical density was measured again at 24 and 48 hours after incubation.

The dilution line for the pipes containing acids was carried out after 24 hours in the incubator.

After completing the dilutions, 0.5 mL were taken from the tubes and then cultivated on a petri dish, adding a thin layer of Agar (Figure 15).

Serial dilutions varied depending on whether they were made on bifidobacteria or clostridia and not all dilutions made in tubes were grown on plates.

Then they were placed under anaerobic conditions and incubated at 37°C for 24 hours.

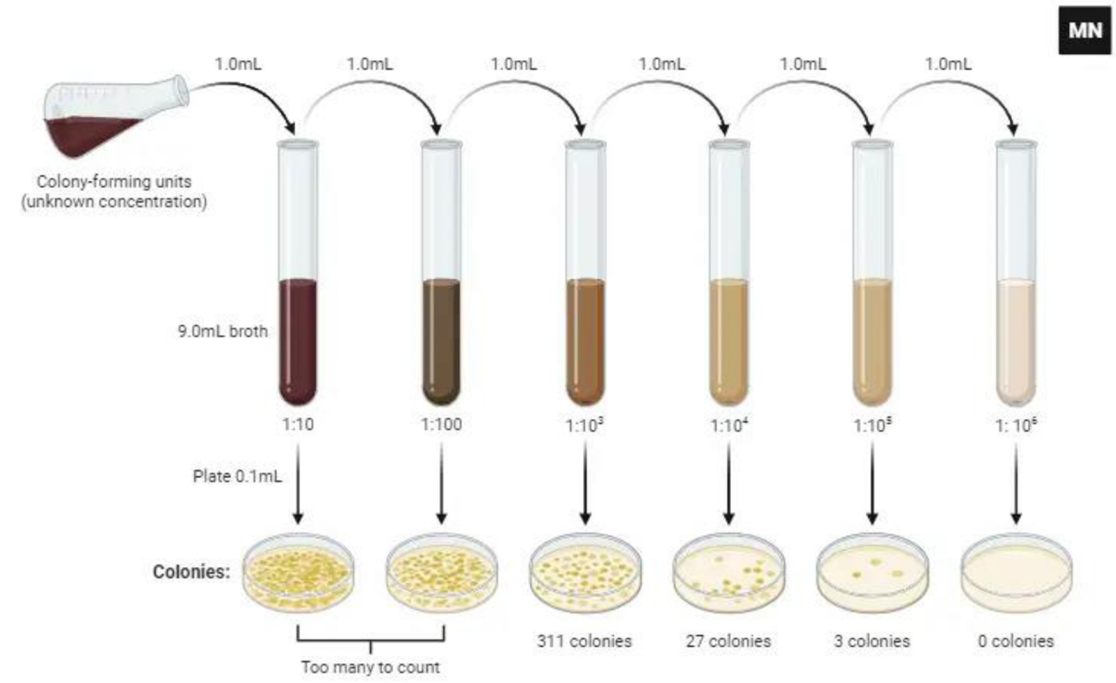

Figure 15 – Dilution line

After 24 hours, I can do microbial counts by reading the petri dishes, as can be seen in Figures 15 and 16.

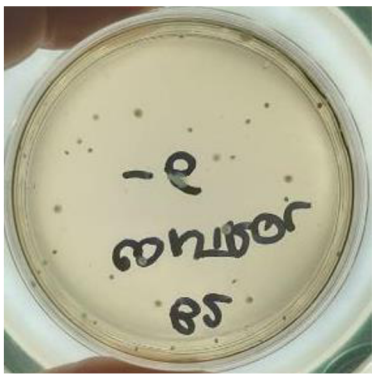

Figure 15

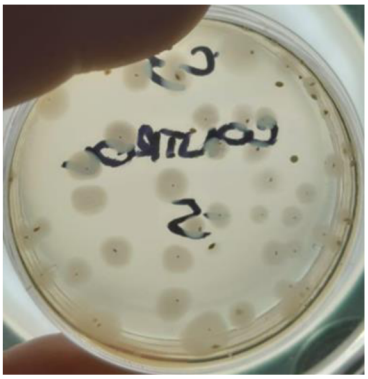

Figure 16

4. Results and Discussion

In total, 38 strains of bifidobacteria and 29 strains of clostridia were screened by the Kirby-Bauer method for the sensitivity to 67 selected antibiotics/discs (65 different antibiotics, one was used in 3-disc concentrations). Inhibition zones were measured in

mm to determine the tested strains for their sensitivity or resistance to used antibiotics with defined concentrations. The sensitivity of selected strains and antibiotics was tested using E-tests. The aim was to validate resistance to mupirocin and norfloxacin and find some other antibiotics that can help determine separately bifidobacteria and clostridia. Similarly, the screening for sensitivity to acetic, formic, lactic, and propionic acid was performed by the diffusion method, and the effect was evaluated using a quantification method when bifidobacterial strains were cultivated on media supplemented by acids with different concentrations compared to media without acids.

4.1 Bifidobacterial and clostridial resistance and sensitivity to tested antibiotics

Due to the high number of used antibiotics and strains, we decided to categorize the results of sensitivity as determined below:

R resistant to the antibiotic dose used (zone 6-7 mm).

R^A resistant to the antibiotic dose used with one susceptible strain observed.

Rx resistant to the antibiotic dose used with few susceptible strains observed.

S sensitive to the antibiotic dose used

S^A sensitive to the antibiotic dose used with one resistant strain observed.

Sx sensitive to the antibiotic dose used with few resistant strains observed.

From 67 used antibiotic discs, we found that tested bifidobacterial strains are resistant to 7 antibiotics (Tobramycin 10 µg, Fluconazole 25 µg, Neomycin 10 µg, Netilmicin 10 µg, Ticarcillin 75 µg, Colistin 10 µg, Polymyxin B 300 IE). As resistant but with one random sensitivity (1 strain from 38 strains) were found Kanamycin 30 µg and Mupirocin 200 µg. However, this can result from some mistake during result reading or contamination, which was not detected during the strain purity testing. Bifidobacteria were found to be resistant with some tested strains -reported (Rx) against 13 antibiotics: Aztreonam 30 µg, Flumequine 30 µg, Nalidixic acid 30 µg, Norfloxacin 10 µg, Oxolinic acid 2 µg, Meropenem 10 µg, Sulphafurazole 300 µg, Pefloxacin 5 µg, Lomefloxacin 10 µg, Mecillinam 10 µg, Gentamicin 10 µg and 13 µg and Amikacin 3 µg. Here the assumption

of strain-specific resistance is possible, however, it would need to be verified with a higher dose or E-test.

The rest of all tested antibiotic discs (45 from 67) were in category sensitive, but we also detected some specific results described below.

As for clostridia, they generally resulted to be more susceptible in particular, clostridia were sensitive to 51 of the 67 antibiotic discs used, and the remaining 16 showed a certain trend of resistance to the dose of the antibiotic used. Partial results are presented in Supplementary Table 2. Clostridia seems to be resistant to Tobramycin 10 µg, Fluconazole 25 µg, Kanamycin 30 µg, Mupirocin 200 µg, Neomycin 10 µg, Netilmicin 10 µg, Aztreonam 30 µg, Ticarcillin 75 µg, Colistin 10 µg, Polymyxin B 300 IE, Sulphafurazole 300 µg, Gentamicin 10 µg, Gentamicin 13 µg, Gentamicin 120 µg, Amikacin 3 µg, Framycetin 100 µg. An obvious sensitivity sensitivity (S, S^A) of clostridia to Streptomycin 300 µg, Amoxycilin 25 µg, Amoxycilin + Clavulanic acid 30 µg, Piperacillin 30 µg, Piperacillin/Tazobactam 36 µg, Cefoperazone 75 µg, Cefovecin 30 µg, Cefoxitin 30 µg, Ceftriaxone 30 µg, Vancomycin 5 µg, Meropenem 10 µg, Azitromycin 15 µg, Erythromycin 15 µg, Spiramycin 100 µg, Nitrofuratoin 100 µg, Chloramphenicol 30 µg, Linezolid 10 µg, Bacitracin 10 IU, Doxycycline 30 µg, Minocycline 30 µg, Oxytetracycline 30 µg, Tetracycline 30 µg, Penicillin G 10 Units, Quinupristin/Dalfopristin 15 µg, Baytril R/Reg tm of Bayer 5 µg, Moxifloxacin 5 µg, Fosfomycin 50 µg were found. Again, species- and strain-specific differences were noted here, and the results are sorted into the above-mentioned subcategories. Overall results for both clostridia and bifidobacteria are presented below in Table 7.

	No.	**Antibiotics**	**BIF**	**CLOS**
1	1	Streptomycin (300 µg)	Sx	S
	2	Tobramycin (10 µg)	R	R
	3	Fluconazole (25 µg)	R	R
	4	Kanamycin (30 µg)	R^A	R^A
	5	Mupirocin (200 µg)	R^A	R
	6	Neomycin (10 µg)	R	R
	7	Netilmicin (10 µg)	R	R^A
2	8	Amoxycilin (25 µg)	S	S
	9	Amoxycilin + Clavulanic acid (30 µg)	S	S

	10	Ampicilin (2 µg)	S	Sx
	11	Ampicilin (10 µg)	Sx	Sx
	12	Ampicilin + Sulbactam (20 µg)	Sx	Sx
	13	Aztreonam (30 µg)	Rx	R
3	14	Doripenem (10 µg)	Sx	Sx
	15	Piperacillin (30 µg)	S	S
	16	Piperacillin/Tazobactam (36 µg)	S	S
	17	Ticarcillin (75 µg)	R	R
	18	Cefaclor (30 µg)	Sx	Sx
	19	Cefepime (30 µg)	Sx	Sx
4	20	Cefoperazone (75 µg)	S	S
	21	Cefovecin (30 µg)	S	S^A
	22	Cefoxitin (30 µg)	S^A	S^A
	23	Ceftaroline (5 µg)	S	Sx
	24	Ceftazidime (30 µg)	Sx	Sx
	25	Ceftibuten (30 µg)	S	Sx
	26	Ceftriaxone (30µg)	S	S
5	27	Cefuroxime (30 µg)	S	Sx
	28	Vancomycin (5 µg)	S	S^A
	29	Flumequine (30 µg)	Rx	Sx
	30	Nalidixic acid (30 µg)	Rx	Sx
	31	Norfloxacin (10 µg)	Rx	Sx
	32	Oxolinic acid (2 µg)	Rx	Sx
6	33	Ertapenem (10 µg)	Sx	Sx
	34	Meropenem (10 µg)	Rx	S^A
	35	Azitromycin (15 µg)	S	S^A
	36	Erythromycin (15 µg)	S	S^A
	37	Lincomycin (10 µg)	S	Sx
	38	Oleandomycin (15 µg)	S	Sx
7	39	Spiramycin (100 µg)	S	S^A
	40	Nitrofuratoin (100 µg)	S	S
	41	Chloramphenicol (30 µg)	S	S
	42	Linezolid (10 µg)	S	S^A
	43	Bacitracin (10 IU)	S	S^A
	44	Colistin (10 µg)	R	R
8	45	Polymyxin B (300 IE)	R	R
	46	Doxycycline (30 µg)	S	S
	47	Minocycline (30 µg)	S	S
	48	Oxytetracycline (30 µg)	S	S
	49	Tetracycline (30 µg)	S	S
9	50	Sulphafurazole (300 µg)	Rx	Rx
	51	Penicillin G (10 Units)	S	S
	52	Quinupristin/Dalfopristin (15 µg)	S	S^A

	53	Pefloxacin (5 µg)	Rx	Sx
	54	Lomefloxacin(10 µg)	Rx	Sx
	55	Mecillinam (10 µg)	Rx	Sx
10	56	Gentamicin (10 µg)	Rx	Rx
	57	Gentamicin (30 µg)	Rx	Rx
	58	Gentamicin (120 µg)	S	Rx
	59	Baytril R/Reg tm of Bayer (5 µg)	Sx	S^A
	60	Clindamycin (2 µg)	Sx	Sx
	61	Nafcillin (1 µg)	Sx	Sx
11	62	Ofloxacin (5 µg)	Sx	Sx
	63	Amikacin (3 µg)	Rx	R
	64	Ciprofloxacin (5 µg)	Sx	Sx
	65	Moxifloxacin (5 µg)	Sx	S^A
	66	Fosfomycin (50 µg)	Sx	S^A
	67	Framycetin (100 µg)	Sx	Rx

Table 7 - Overall results for both clostridia and bifidobacteria

From which it follows that in both cases the resistance of bifidobacteria to Mupirocin (200 µg), which is a common component of bifidobacterial media, was confirmed (Rada, 2000; Rada, 1999). However, clostridia are also resistant, which was indicated by previously published results that dealt with, for example, the isolation of bifidobacteria or clostridia from fecal samples of various animals (Makovska, 2023). Vlková *et al*. (2015) indicate that a medium modified with the addition with norfloxacin (Vlkova, 2015) may be more effective in selection focused especially on bifidobacteria, however strain-specific sensitivity to norfloxacin was detected here as well. Even though clostridia are more sensitive to Norfloxacin (10 µg) according to our results, there are also resistant strains, however the used concentration is lower than those recommended for the medium. Flumequine, Nalidixic acid, Norfloxacin, Oxolinic acid, Pefloxacin, and Lomefloxacin belong to (fluoro)quinolone antibiotics, and some of the tested strains of bifidobacteria and clostridia showed the same trend in sensitivity to this group of antibiotics. At least there is an interesting sensitivity of only some bifidobacteria and further research is desirable.

Thus, when comparing the resistances and sensitivities of bifidobacteria and clostridia, it can be observed that most strains of both taxa were resistant to Tobramycin 10 µg,

Fluconazole 25 µg, Kanamycin 30 µg, Mupirocin 200 µg, Neomycin 10 µg, Netilmicin 10 µg, Aztreonam 30 µg, Ticarcillin 75 µg, Colistin 10 µg, Polymyxin B 300 IE, Amikacin 30 µg. This could be a starting point for the selection of antibiotics to be used in media designed for the recovery of both genera. However, some doses were low, and the use of higher concentration would probably lead to different results. This was observed in the case of gentamicin, which was used in 3 different doses (10, 30, and 120 µg). Empolying 10 and 30 µg clostridia and bifidobacteria resulted resistant but using 120 µg bifidobacteria were sensitive and clostridia showed strain specific differences (Rx), and some strains were sensitive. Although it is known that bifidobacteria may harbour tetracycline resistance genes (Wang, 2017) in their genome, we did not record resistance in any of the 38 bifdobacterial strains. As for clostridia the same situation was observed.

Based on how the antibiotics are divided, it should be possible to analyse only one antibiotic in the group, to determine the sensitivity or resistance of the entire group. However, if we look at Supplementary Table 1 and 2, we can observe that this is actually not possible.

After carefully analyzing the screening results through Kirby-Bauer, it was possible to select the antibiotics and strains that were going to be used for the E-test. Strains that had reported abnormal results compared with the rest of the population were chosen, as well as strains that had results consistent with the rest of the microbial population. Five bifidobacteria were selected*: B. animalis* subsp. *lactis* (B005), *B. adolescentis* (B012*), B. catenulatum* subsp. *kashiwanohense* (B015), *B. breve* (B019) and *Bifidobacterium* sp. *nov. VB17* (B029) with regard to bifidobacterial together with 5 clostridia, *C. perfringens* (C007), *C. neonatale* (C008), *C. butyricum* (C015), *Paeniclostridium sordelli* (C017) and *Cl. difficile* (C028).

Three antibiotics were used for the E-test: Nalidixic acid, Ceftibuten, and Mecillinam. Disc test zone 6 mm = resistance to tested concentration.

	B005	B012	B015	B019	B039	C007	C008	C015	C017	C028
Nalidixic acid E-test										
Disc test 30 µg (zone mm)	6	6	15	6	6	13	15	22	15	6
E-test (MIC)	**R**	**R**	12	**R**	**R**	24	6	6	**R**	**R**
Ceftibuten										
Disc test 30 µg (zone mm)	6	6	6	6	6	11	6	6	18	6
E-test (MIC)	**R**	**R**	**R**	**R**	**R**	0.004	**R**	**R**	1,5	**R**
Mecillinam										
Disc test 10 µg (zone mm)	6	6	6	6	6	6	6	6	15	6
E-test (MIC)	24	8	96	96	8	12	6	6	0.75	**R**

Table 8 – Result E-test

The obtained results from the E-test method (see Table 8) were not fully corresponding with disc method (Kirby-Bauer). In case of Nalidixic acid, the results of disc method and E-test were different only one clostridial strain (*Paeniclostridium sordelli* C017). Results of ceftibuten testing were fully correlated with both methods. The biggest differences were detected with mecillinam, when minimal inhibition concentration (MIC) was within range 0.75 – 96, and one strain was resistant. The use disc concentration was 10 µg. The strain (*Paeniclostridium sordelli* C017) with MIC 0.75 had inhibition zone to mecillinam 15 mm and the strain (*Cl. difficile* C028) without zone was by E-test resistant, what seems to be right. However, all other strains were resistant to the concentration 10 µg, but their MIC range among 6 – 96, what was strange.

The results indicate the need for optimization of testing (increase the number of repetitions) and supplementation with control strains.

4.2 Effect of selected acids on bifidobacterial and clostridial growth

Acid resistance had to be tested, so both bifidobacteria and clostridia were grown on Petri dishes, through the diffusion method they came into contact with the 4 selected acids (propionic acid, formic acid, acetic acid and lactic acid) each at 3 different concentrations (0.5%, 1% and 2%) as described in the methodology. All results of this screening are presented in Supplementary Table 3 and 4. After incubating them for 24 hours, the results were ready, and halos were measured (in the same way done for antibiotics). Almost all species of bacteria at almost all acid concentrations were found to be resistant. As for bifidobacteria, no species were found to be sentivie to most of the

acid type and concentration. Only in case of formic acid in 2% concentration some strains were evaluated as sensitive. On the other hand, it can be observed that clostridia were sensitive to 2% concentration of propionic, formic and acetic acids; only a few species of clostridia were sensitive to 2% concentration of lactic acid, while the clostridia are mostly resistant with acid concentration at 0.5% and 1%. Bifidobacteria do not belong to lactic acid bacteria group, but they have specific metabolic pathway, thus, they produce mainly acetic acid, after lactic acid (generally in ration 3:2), and formic acid is produced in minor concentrations (Russell, 2011). This is one of the main mechanisms exploited for the inhibition of pathogenic bacteria such as clostridia (Aw, 2019; Wei, 2018). Which, according to the results found, could be used to increase the selectivity of the media and suppress clostridia.

The effect of acids on the growth of bifidobacterial and clostridial species was also evaluated using their cultivation in broth media with acids. In Table 9 are results of after 24 h and 48 h cultivation. The used strain of bifidobacteria and clostridia were the offered detected species; *B. animalis* (B1 = B005), *B. breve* (B2 = B019), *C. butyricum* (C1 = C033), *Cl. difficile* (C2 = C028) and *C. perfringens* (C3 = C007). The results indicate that the increased concentration of all used acids influenced the growth and density of bacterial cultures, namely bifidobacteria and clostridia. Here, differences were detected between the tested species and individual acids.

DENISITY																			
Acids	% v/v	B1			B2			C1			C2			C3					
		t=0	t=24h	t=48h	**t=0**	t=24h	t=48h	**t=0**	t=24h	t=48h	**t=0**	t=24h	t=48h	**t=0**	t=24h	t=48h			
Acetic acid	0.25	0.36	4.46	5.1	1.34	3.58	5.2	0.27	1.05	1.13	0.47	0.51	0.74	1.8	1.83	2			
	0.5	0.34	1.03	1.3	1.25	1.83	1.99	0.25	1.11	1.16	0.81	0.81	0.9	2.03	2.29	2.98			
	1	0.21	0.8	0.8	1.33	1.33	1.33	0.44	1.23	1.28	0.91	0.91	0.91	2.27	2.69	3.17			
Lactic acid	0.25	0.7	1.92	2.04	1.24	2.91	4.4	0.35	1.01	1.05	0.5	0.54	0.97	1.59	1.62	7			
	0.5	0.71	1.14	1.26	1.18	1.5	1.42	0.29	1	1.08	0.83	0.9	0.96	1.9	2.25	2.57			
	1	0.84	1.06	1.06	1.19	1.2	1.33	0.42	1.3	1.46	0.51	0.62	1.2	2.13	2.75	3.6			
Formic acid	0.25	0.45	1.26	1.41	1.31	1.31	1.31	0.37	1.58	1.76	0.61	0.9	1	1.6	2.25	2.35			
	0.5	0.44	1.43	1.49	1.41	1.41	1.41	0.21	1.6	1.8	0.9	0.9	1.3	2.18	2.85	3.6			
	1	0.32	1.36	1.42	1.34	1.39	1.4	0.79	1.86	1.85	0.78	0.8	1.42	2.47	3.26	2.84			
Propionic acid	0.25	1.24	5.85	5.53	1.36	5.31	6.29	0.27	1.01	0.83	0.54	0.56	0.72	1.93	2.11	6.09			
	0.5	0.39	1.46	1.38	1.2	2.02	2.8	0.27	1.07	1.14	0.4	0.5	0.77	1.9	1.93	2.02			
	1	0.37	1.6	1.43	1.17	1.7	1.68	0.25	1.26	1.34	0.7	0.73	0.87	2.04	2.1	2.3			
CONTROL		0.89	10.8	11.5	1.28	10.1	10	0.92	4.31	4.48	0.43	5.11	5.73	2.14	9.77	6.44			

Table 9 – Optical density

Given the scanty results obtained by the analysis of bifidobacteria and clostridia on Petri dishes with the diffusion method, it was decided to further investigate effect of acids in agar media on detected counts of bacteria after cultivation. Only two species of bifidobacteria and three species of clostridia were examined during this more elaborate part of the experiment. It was decided to use three species of clostridia instead of two because earlier in the experiment the first species analyzed yielded no results. Prior to

plate culture, optical density was measured at time 0, after 24 hours and after 48 hours after inoculation of the bacteria into the tubes containing the culture medium and the different acid concentrations maintained inside the incubator at 37°C. Bifidobacteria were cultivated from time zero to time 48h, in particular at of 0.25 percent of all acid types.

As for the clostridia, the experiment with the first species found no growth; it was possible to observe this from the optical density values. The other two species of clostridia were found to be quite sensitive to acids.

Table 10 shows the detected numbers of the tested bacterial strains after 24 h of cultivation, when 1 ml of the freshly grown bacterial culture was diluted using ten-fold dilution. Individual dilutions were then cultured to determine bacterial abundance as log CFU/ml. In order to be able to evaluate the effect of the addition of acid of a certain concentration in the medium on the detected numbers of bacteria. Cultivation was thus carried out with different media, i.e. acid (acetic, lactic, formic, propionic) of concentration (0.25% and/or 0.5%). The results show that the addition of acids significantly affected the number of detected bacteria. In the medium commonly used for the detection of bifidobacteria, the acetic acid content is 1 ml/1L, which corresponds to a concentration of 0.1%. Increasing to 0.25% concentration did not significantly affect bifidobacteria counts, but there was a significant reduction in clostridia counts. A concentration of 0.5% already affected the numbers of all tested strains, both bifidobacteria and clostridia. For the other acids, the results were varied, depending on the tested strain, again a concentration of 0.5% was too much. the concentration of 0.25% propionic acid showed interesting results, when it suppressed the growth of clostridia, but not of bifidobacteria. To increase the selectivity of the bifidobacterial medium, it is possible to increase the concentration of acetic acid or add propionic acid. However, more species and strains of both bifidobacteria and clostridia need to be tested. Strain C1 showed often poor growth in the lab conditions, so the results are not relevant.

	B1	B2	C1	C2	C3
CONTROL t=0	7.49 log CFU/mL	7.56 log CFU/mL	7.25 log CFU/mL	5.63 log CFU/mL	7.11 log CFU/mL
Acetic Acid 0.25%	7.70 log CFU/mL	7.48 log CFU/mL	0 CFU/mL	4.58 log CFU/mL	5.69 log CFU/mL
Acetic Acid 0.5%	5.89 log CFU/mL	4.48 log CFU/mL	0 CFU/mL	3.07 log CFU/mL	5.80 log CFU/mL
Lactic Acid 0.25%	6.84 log CFU/mL	7.64 log CFU/mL	0 CFU/mL	4.82 log CFU/mL	5.91 log CFU/mL
Lactic Acid 0.5%	3.43 log CFU/mL	7.63 log CFU/mL	0 CFU/mL	0 CFU/mL	2.94 log CFU/mL
Formic Acid 0.25%	0 CFU/mL	7.56 log CFU/mL	0 CFU/mL	2.10 log CFU/mL	2.85 log CFU/mL
Formic Acid 0.5%	0 CFU/mL	7.02 log CFU/mL	0 CFU/mL	1.55 log CFU/mL	0 CFU/mL
Propionic Acid 0.25%	7.55 log CFU/mL	6.67 log CFU/mL	0 CFU/mL	0 CFU/mL	0 CFU/mL
Proprionic Acid 0.5%	6.54 log CFU/mL	7.63 log CFU/mL	0 CFU/mL	0 CFU/mL	0 CFU/mL

Table 10 - Numbers of the tested bacterial strains after 24 h of cultivation

The sensitivity of clostridia to acids was presented in more studies. According to Wrigley (1995), when large amounts of *C. perfringens* are consumed and it sporulates (releases enterotoxin into the intestine), it can cause gastroenteritis. The organism's ability to form spores may be impacted by the acidic environment of the stomach because it must pass through it. After being subjected to acidic conditions, five strains of *C. perfringens* were assessed for survival and spore-forming capacity. The bacteria eventually perish in an acidic pH environment, but the remaining cells can regenerate and produce spores. A 30-minute exposure to a pH 2 environment resulted in increased sporulation in two of the five strains. Enterotoxin concentrations were higher in acid-exposed cells compared to untreated cells for four of the tested strains. The bacteria that could withstand acid and sporulation the best were those in the stationary phase of growth. The findings suggest that when exposed to an acidic environment, certain strains will produce more enterotoxins and spores.

Furthermore, more recent research has demonstrated that citric acid, oleic acid, and linoleic acid can all inhibit for example *Cl. difficile* strains (Skrivanova, 2006); lastly, a different study found that lactic acid inhibited *Cl. difficile* more successfully than acetic acid (Huertas-Diaz, 2023).

5. Conclusion

The initial aim of the thesis was to do screening different species of bifidobacteria and clostridia to find a more selective medium with the help of antibiotics and acids. We were able to do a thorough screening with 65 antibiotics and 4 acids that showed us the

resistance and sensitivity of across various species and strains of bifidobacteria and clostridia. We confirmed that bifidobacterial as well as clostridia are resistant to mupirocin. However, their clostridial sensitivity to norfloxacin seems to be variable, and not all bifidobacterial are resistant. By performing E-test, it was possible to confirm the resistance of some strains, but some results were different, so more tests, repetition and optimization of the methodology would be appropriate.

Despite the large number of tested antibiotics, no suitable antibiotics were found that would allow supplementing the mupirocin medium for selective quantification of either bifidobacteria or clostridia. However, increasing the content of lactic or acetic acid seems to be a suitable solution for increasing the selectivity of the bifidobacterial mupirocin medium due to clostridial inhibition. Finding a suitable component for the selection of clostridia requires further research, where it would be useful to connect our new findings and verified selectivity factors in media for clostridia.

Supplementary Materials

Description: The accompanying Excel spreadsheet shows the results regarding the screening performed on bifidobacteria and clostridia by diffusion with antibiotics and acids.
Filename: Supplementary Material - Melosi Claudia

Bibliography

Ammor M., Florez A., Hoek A., Los Reyes-Gavilan C., Aarts H., Matgolles A., Mayo B. 2008. Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacterial. J Mol Microbiol Biotechnol. DOI: 10.1159/000106077

Arboleya S., Watkins C., Stanton C., Ross P. 2016. Gut Bifidobacteria Populations in Human Health and Aging. Front Microbiol. DOI: 10.3389/fmicb.2016.01204

Balouiri M., Sadiki M., Koraichi Ibnsouda S. 2016. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. doi: 10.1016/j.jpha.2015.11.005

Bredius M.W.J. Ree E.M. 2003. Chapter 3 Media for the detection and enumeration of clostridia in foods. Progress in Industrial Microbiology. https://doi.org/10.1016/S0079-6352(03)80006-1

Brook I., Frazier E. H. 1993. Significant Recovery of Nonsporulating Anaerobic Rods from Clinical Specimens. Clinical Infectious Diseases. https://doi.org/10.1093/clind/16.4.476

Chen J., Chen X., Ho C. 2021. Recent Development of Probiotic Bifidobacteria for Treating Human Diseases. Frontiers in Bioengineering and Biotechnology.

Chai C., Lee K., Lee D., Lee S., Oh S. 2015. Non-selective and selective enrichment media for the recovery of Clostridium difficile from chopped beef. Journal of Microbiological Methods. https://doi.org/10.1016/j.mimet.2014.12.001

Cronin M., Ventura M., Fitzferald G., Sinderen D. 2011. Progress in genomics, metabolism and biotechnology of bifidobacterial. Int J Food Microbiol. DOI: 10.1016/j.ijfoodmicro.2011.01.019

De Jong A.E.I., Eijhusen G.P, Brouwer-Post E.J.F, Grand M., Johansson T., Kärkkäinen T., Marugg J., Veld P.H., Warmerdam F.H.M., Wörner G., Zicavo A., Rombouts F.M., Beumer R.R. 2003. Comparison of media for enumeration of Clostridium perfringens from foods. Journal of Microbiological Methods. https://doi.org/10.1016/S0167-7012(03)00069-1

Duranti S, Lugli GA, Milani C et al. 2019. Bifido- bacterium bifidum and the infant gut microbiota: an intriguing case of microbe-host co-evolution. Environ Microbiol 2019.

Duranti S., Longhi G., Ventura M., Sinderen D., Turroni F. 2020. Exploring the Ecology of Bifidobacteria and Their Genetic Adaptation to the Mammalian Gut. Microorganisms 2021. https://doi.org/10.3390/microorganisms9010008

Durre P. 2014. Physiology and Sporulation in Clostridium. Microbiol Spectr. DOI: 10.1128/microbiolspec.TBS-0010-2012

Egan M., Dempsey E., Ryan A., RossP., StantonaC. 2021. The Sporobiota of the Human Gut. Gut Microbes. doi: 10.1080/19490976.2020.1863134

Fatima S., Altwaijry H., Abulmeaty M., Abudawood M., Siddiqi N., Alrashoudi R. H., Alsobaie S. 2023. Combined Supplementation of Clostridium butyricum and Bifidobacterium infantis Diminishes Chronic Unpredictable Mild Stress-Induced Intestinal Alterations via Activation of Nrf-2 Signaling Pathway in Rats. Int J Mol Sci. doi: 10.3390/ijms24098264

Ferraris L., Butel M. et al. 2012. Clostridia in Premature Neonates' Gut: Incidence, Antibiotic Susceptibility, and Perinatal Determinants Influencing Colonization. PLoS Uno. doi:10.1371/journal.pone.0030594

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

Gao J., Li X., Zhang G., Sadiq F., Simal-Gandara J., Xiao J., Sang Y. 2021. Probiotics in the dairy industry-Advances and opportunities. Compr Rev Food Sci Food Saf. DOI: 10.1111/1541-4337.12755

Gomes A. M. P., Malcata, F. X. 1999. Bifidobacterium spp. and Lactobacillus acidophilus: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends in Food Science & Technology. https://doi.org/10.1016/S0924-2244(99)00033-3

Guo P., Zhang K., Ma X., He P. 2020. Clostridium species as probiotics: potentials and challenges. Journal of Animal Science and Biotechnology.

Hidalgo-Cantabrana C., Delgado S., Ruiz L., Ruas-Madiedo P., Sanchez B., Margolles A. 2017. Bifidobacteria and Their Health-Promoting Effects. Microbiology Spectrum. https://doi.org/10.1128/microbiolspec.bad-0010-2016

Hill C., Guarner F., Reid G., Gibson G., Merenstein D., Pot B., Morelli L., Berni Canani R., Flint H., Salminen S., Calder P., Sanders M. 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology.

Hurtyas-Diaz L., Kyhnau R. et al. Breastfeeding and the major fermentation metabolite lactate determine occurrence of Peptostreptococcaceae in infant feces. 2023. Gut Microbes. doi: 10.1080/19490976.2023.2241209

Humanitas Research Hospital. www.humanitas.it. Consulted on 26/09/2023.

Hungate, R.E. and J. Macy. 1969. The roll-tube method for cultivation of strict anaerobes. Bulletins from the ecological research committee.

Infezioni Obiettivo zero. www.infezioniobiettivozero.info. Consulted on 12/10/2023.

International Organization for Standardization. www.iso.org. Consulted on 23/10/2023.

Italian Ministry of Health. www.salute.gov.it. Consulted on 21/10/2023.

Judkins T., Archer D., Kramer D., Solch R. 2020. Probiotics, Nutrition, and the Small Intestine. Current Gastroenterology Reports.

Kawasaki S., Mimura T., Satoh T., Takeda K., Niimura Y. 2006. Response of the Microaerophilic Bifidobacterium Species, B. boum and B. thermophilum, to Oxygen. Appl Environ Microbiol. doi: 10.1128/AEM.01216-06.

Koc J., Rada V.. 2000. The use of mupirocin for selective enumeration of bifidobacteria in fermented milk products. Milchwissenschaft.

Koopman N., Remijas L., Seppen J., Setlow P., Brul S. 2022. Mechanisms and Applications of Bacterial Sporulation and Germination in the Intestine. Int J Mol Sci.

Korpela K., Salonen A., Vepsäläinen O. et al. 2018 Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. Microbiome.

Lawson P. 2016. The taxonomy of the genus Clostridium: current status and future perspectives. Microbiology China.

Lecorn D., Vertucci F. et al. 2007. In Vitro Activity of Amoxicillin, Clindamycin, Doxycycline, Metronidazole, and Moxifloxacin Against Oral Actinomyces. Journal of Endodontics. https://doi.org/10.1016/j.joen.2007.02.002

Li Q., Chen Q., Ruan H., Zhu D., He G. 2010. Isolation and characterisation of an oxygen, acid and bile resistant Bifidobacterium animalis subsp. lactis Qq08. J Sci Food Agric. DOI: 10.1002/jsfa.3942

Ling Z., Liu X., Cheng Y., Luo Y., Yuan L., Li L., Xiang C. 2015. Clostridium butyricum combined with Bifidobacterium infantis probiotic mixture restores fecal microbiota and attenuates systemic inflammation in mice with antibiotic-associated diarrhea. Biomed Res Int. DOI: 10.1155/2015/582048

Lugli G., Milani C., Turroni F., Duranti S., Mancabelli L., Mangifesta M., Ferrario C., Modesto M., Mattarelli P., Jiri K., Sinderan D., Ventura M. 2017. Comparative genomic and phylogenomic analyses of the Bifidobacteriaceae family. BMC Genomics. DOI: 10.1186/s12864-017-3955-4

Makovska M., Modrackova N., Bolechova P., Drnkova B., Neuzil-Bunesova V. 2021. Antibiotic susceptibility screening of primate-associated Clostridium ventriculi. Anaerobe. https://doi.org/10.1016/j.anaerobe.2021.102347

Manno G., Ugolotti E. et al. 2003. Use of the E test to Assess Synergy of Antibiotic CombinationsAgainst Isolates of Burkholderia cepacia-Complex from Patientswith Cystic Fibrosis. Eur J Clin Microbiol Infect Dis. DOI 10.1007/s10096-002-0852-8

Microbiologia Italia. www.microbiologiaitalia.it. Consulted on 21/10/2023.

Nakajo K., Takahashi N., Beighton D. 2010. Resistance to Acidic Environments of

Caries-Associated Bacteria: Bifidobacterium dentium and Bifidobacterium longum. Caries Research. https://doi.org/10.1159/000318582

Nunziata L., Brasc M., Morandi S., Silvetti T. 2022. Antibiotic resistance in wild and commercial non-enterococcal Lactic Acid Bacteria and Bifidobacteria strains of dairy origin: An update. Food Microbiol. DOI: 10.1016/j.fm.2022.103999

Nuriel-Ohayon M., Neuman H., Ziv O et al. 2019. Progesterone Increases Bifidobacterium Relative Abundance during Late Pregnancy. Cell Rep 2019.

O'Callaghan A., Sinderen D. 2016. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. Front Microbiol. DOI: 10.3389/fmicb.2016.00925.

Odamaki T., Kato K., Sugahara H., Hashikura N., Takahashi S., Xiao J., Abe, Osawa R. 2016. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol. Doi: 10.1186/s12866-016-0708-5

O'Grady K., Cavaliere D., Riley T. 2021. Antimicrobial resistance in Clostridioides difficile. ur J Clin Microbiol Infect Dis. DOI: 10.1007/s10096-021-04311-5

Popoff M. R. 1984. Selective medium for isolation of Clostridium butyricum from human feces. J Clin Microbiol. doi: 10.1128/jcm.20.3.417-420.1984

Popoff M., Bouvet P. 2009. Clostridial toxins. Future Microbiol. doi: 10.2217/fmb.09.72.

Poupard J., Husain I., Norris R. 1973. Biology of the bifidobacterial. Bacteriol Rev. doi: 10.1128/br.37.2.136-165.1973

Rahman, Md. M., Kim, W.-S., Kumura, H., & Shimazaki, K. 2008. Autoaggregation and surface hydrophobicity of bifidobacteria. World Journal of Microbiology and Biotechnology. https://doi.org/10.1007/s11274-007-9650-x

Ríos-Covián D., Ruas-Madiedo P., Margolles A., Gueimonde M., G. de los Reyes-Gavilán C., Salazar N. 2016. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Front Microbiol. Doi: 10.3389/fmicb.2016.00185

Roy D. 2003. Chapter 10 Media for the detection and enumeration of bifidobacteria in food products. Progress in Industrial Microbiology. https://doi.org/10.1016/S0079-6352(03)80013-9

Sharma R. 2022. Kirby Bauer Disc Diffusion Method For Antibiotic Susceptibility Testing.

Shaw A., Cornwell E. et al. 2020. Dynamics of toxigenic Clostridium perfringens colonisation in a cohort of prematurely born neonatal infants. BMC Pediatr. doi: 10.1186/s12887-020-1976-7

Shen A., Edwards A., Sarker M., Paredes-Sabjae D. 2019. Sporulation and Germination in Clostridial Pathogens. Microbiol Spectr. doi: 10.1128/microbiolspec.GPP3-0017-2018

Skirivanova E., Marounek M., Benda V., Brezina P. 2006. Susceptibility of Escherichia coli, Salmonella sp. and Clostridium perfringensto organic acids and monolaurin. Vet Med.

Slavin J. 2013. Fiber and Prebiotics: Mechanisms and Health Benefits. Nutrients. Doi: 10.3390/nu5041417

Spigaglia P., Mastrantonio P., Barbanti F. 2018. Antibiotic Resistances of Clostridium difficile. Adv Exp Med Biol. DOI: 10.1007/978-3-319-72799-8_9

Stevens D., Aldape M., Bryant A. 2012. Life-threatening clostridial infections. Anaerobe. https://doi.org/10.1016/j.anaerobe.2011.11.001

Synalab Dott. Alexander Bertuccioli. Bifidobaterium: il come, il quando e il perché di un genere batterico che si trasmette da madre a figlio. www.synalab.it Consulted on 24/10/2023

Tamime A. Y. 1999. MICROFLORA OF THE INTESTINE | Biology of Bifidobacteria. Encyclopedia of Food Microbiology. https://doi.org/10.1006/rwfm.1999.1035

Tascini C., Sozio E., Viaggi B., Meini S. 2016. Reading and Understanding an Antibiogram. Italian Journal of Medicine. DOI: 10.4081/itjm.2016.794

Vlková E., Salmonová H., Bunešová V., Geigerová M., Rada V., Musilová S. 2015. A new medium containing mupirocin, acetic acid, and norfloxacin for the selective cultivation of bifidobacterial. Anaerobe.

Wrigley D., Hanwella H., Thon B. Acid exposure enhances sporulation of certain strains of Clostridium perfringens. 1995. Anaerobe. DOI: 10.1006/anae.1995.1025

https://www.biomerieux.it/ - Consulted on 21/10/2023.