Interactions among yeasts and probiotic bacteria and inhibition of the growth of Bacillus clausii by Debaryomyces hansenii

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INTERACTIONS AMONG YEASTS AND PROBIOTIC BACTERIA AND INHIBITION OF THE GROWTH OF *Bacillus clausii* BY *Debaryomyces hansenii*

This study was carried out under mentorship of Renata Teparić, PhD, Full professor (Faculty of Food Technology and Biotechnology, University of Zagreb), at the Department of Food Microbiology and Biotechnology of Faculty of Food Science at the Hungarian University of Agriculture and Life Sciences under supervision of Ágnes Belák, PhD, Associate professor and with the assistance of Mónika Kovács, PhD, Associate professor.

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INTERACTIONS AMONG YEASTS AND PROBIOTIC BACTERIA AND INHIBITION OF THE GROWTH OF *Bacillus clausii* BY *Debaryomyces hansenii*

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Abstract: Cross-kingdom interactions exist in many environments, including various human body niches. In order to understand these interactions better, 16 yeasts were isolated from the pear (with some rotten spots) and five bacteria cultures were isolated from the probiotic supplements. Yeast isolates were inoculated on 15 media in order to determine the optimal growth conditions for each of them. Bacteria isolated from the probiotic preparation were identified by MALDI-TOF, while the cell morphology of the yeasts was observed under the microscope. Relationships among isolated yeasts and probiotic bacteria were evaluated by contact-inhibition test, the cell-free cultivation media test and co-culturing test. According to the results of the contact-inhibition test, yeasts B5 and B10 inhibited the growth of *Bacillus clausii*. Both inhibitory yeast isolates were identified as *Debaryomyces hansenii* by 28 rDNA sequencing. This unique finding was confirmed with co-culturing of *B. clausii* and *D. hansenii* that also described how this relationship is strain-dependent.

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INTERAKCIJE IZMEĐU KVASACA I PROBIOTIČKIH BAKTERIJA TE INHIBICIJA RASTA *Bacillus clausii* S *Debaryomyces hansenii*

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Sažetak: Interakcije među pripadnicima različitih carstava su prisutne u mnogim staništima, uključujući različite organske sustave ljudskog tijela. Kako bi se bolje razumjele ove interakcije, 16 vrsti kvasaca je izolirano iz djelomično trule kruške i 5 bakterijskih vrsti je izolirano iz probiotičkih pripravaka. Izolati kvasaca su nacijepljeni na 15 hranjivih podloga kako bi se odredili optimalni uvjeti uzgoja za svakoga od njih. Bakterije izolirane iz probiotičkog pripravka su identificirane pomoću MALDI-TOF, dok je morfologija kvasaca potvrđena pomoću svjetlosnog mikroskopa. Interakcije između izoliranih kvasaca i probiotičkih bakterija su provjerene s nekoliko testova, uključujući kontaktni test inhibicije, test sa medijima u kojima su mikroorganizmi uzgajani te test zajedničkog uzgoja. Prema rezultatima kontaktnog testa inhibicije kvasci B5 i B10 su inhibirali rast bakterije *Bacillus clausii*. Oba inhibirajuća kvašćeva izolata identificirana su kao *Debaryomyces hansenii* 28 rDNA sekvencioniranjem. Ovo jedinstveno otkriće potvrđeno je zajedničkim uzgojem *B. clausii* i *D. hansenii* te je zaključeno kako je ova inhibicija ovisna o soju kvasca.

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Contents

1. INTRODUCTION

Single-celled organisms were the very first life forms which appeared on Earth. In the beginning, they were prokaryotes in the first oceans, and later they evolved and spread to other habitats. As a result, microorganisms are very diverse and are represented in the three domains of life, Archaea, Bacteria, and Eukarya. Today, they are inevitable inhabitants of different environments, including various foods and multicellular organisms (Wallace et al., 2011). The assemblage of all present microorganisms in a defined environment is known as microbiota. Microbiome, on the other hand, refers to the collection of genomes from all the microorganisms in the specific surrounding (Richard and Sokol, 2019).

The most numerous members of the human microbiota are the bacteria (estimated at between 75 and 200 trillion individual cells). Every part of the human body lives in symbiosis with its microbiome (e.g. skin, respiratory tract, urogenital tract), but the central part of this microbiota is the gastrointestinal tract (GIT). Research in past decades has shown that besides bacteria, yeasts contribute to the balance of the digestion tract in numerous ways. Therefore, it is essential to understand the interactions between these two groups of microorganisms in order to preserve harmony. Microbes are not only commensals that control the population of pathogenic bacteria but also affect the host's health, helping with digestion, inflammation, intestinal integrity and the immune system (Janssens et al., 2018).

Live microorganisms, both bacteria and yeasts, that benefit human health and GIT balance, are called probiotics. They are naturally present or ingested by food or supplements. However, after they are applied to the organism, their maintenance depends on their interaction with already present microbes in the gut (Kim et al., 2019).

The aim of this study was to gain more understanding of interactions among yeasts and probiotic bacteria. To do so, yeasts and probiotic bacteria were isolated from several samples of food and commercial probiotic supplements using the selective media. Isolates were grouped based on colony and cell morphological characteristics and identified by MALDI-TOF MS analysis. Their interactions were examined by contact-inhibition assay and the cell-free cultivation media test. To confirm the results obtained in the contact-inhibition assay, certain isolates were co-cultured and identified by DNA sequencing.

The second goal of this research was to determine the optimal growth conditions for each yeast isolate. Therefore, 48-h-old yeast cultures were inoculated on 15 different growth media and their growth was compared.

2. LITERATURE REVIEW

2.1. FUNGI-BACTERIA RELATIONSHIPS

All ecological communities consist of different species that coexist in a particular area. Interactions between them determine the growth of their populations and abundance. Interactions are categorised depending on the effect on participants. Generally, consequences can be positive (+), negative (-) or neutral (0). Regarding that fact, five main interactions are defined: mutualism, commensalism, amensalism, parasitism and competition (Table 1.). On the other hand, the general term symbiosis, which describes two species who live together in a long-term association, is sometimes misunderstood, not considering that this relationship brings a variety of positive or negative effects on the participants. Three primary types of symbiosis are mutualism, commensalism and competition (Richard and Sokol, 2019).

Table 1. Definitions of interactions between species

NAME	DESCRIPTION	EFFECT
Mutualism	Both microorganisms benefit from each other	+/+
Commensalism	One microorganism benefits from their relationship, and the other is unaffected	+/0
Amensalism	One microorganism negatively affects the other and stays unaffected	-/0
Parasitism	One microorganism benefits and the other is harmed	+/-
Competition	Both organisms use the same resource and negatively impact each other	-/-

Most researches on human GIT microbiota are conducted to understand better bacterial and fungal communities of patients with Crohn's disease. Dysbiosis present, in this case, results from specific interactions among microorganisms. It is discovered that the yeast *Candida tropicalis* and bacteria *Serratia marcescens* or *Escherichia coli* live in association beneficial for both of them. Namely, bacteria and fungi create mixed biofilms larger than biofilms formed of separate microorganisms. So, this interkingdom correlation is a good example of mutualism (Hoarau et al., 2016). The best illustration of commensalism is a relation between fungi *S. cerevisiae* and bacteria *Lactobacillus plantarum* or *Lactococcus lactis*. Specifically, this yeast

secretes amino acids that enable the growth of bacteria, while bacteria produce lactose used by fungi as a carbon source (Ponomarova et al., 2017).

The most interesting type of interplay is surely competition. It has been known since the discovery of penicillin. In the case of gut microbiota, most studies are concerned with the impact of various bacterial strains on the yeast *Candida albicans*. Multiple mechanisms are reported, mainly about the effect on biofilm formation, filamentation and adherence. Some bacteria can even effect on survival of the filament form of this yeast. On the other hand, it is proven that yeast biofilms sometimes protect bacteria in GIT. Primary modes of cross-kingdom interactions involve physical or chemical interplay, modulation of environment or host, competition for nutrients or adhesion sites, and formation of mixed species biofilms.

The effect of bacteria on yeasts in the gut has been a trendy topic in the last decades, but it is still very little known about the negative impact of yeasts on bacteria. Moreover, many kinds of research are conducted *in vitro,* which is not always a reliable prediction of behaviour in the human host. So, this topic is very current and prospective (Krüger et al., 2019).

2.2. GROWTH INHIBITION OF YEASTS BY PROBIOTIC BACTERIA

The most prominent fungus present in the human gut is *Candida albicans*. Because of its frequency and simple cultivation, it is considered a model organism for examining fungal-host and, more importantly, fungal-bacterial interactions. As it can be assumed, this yeast is a symbiotic microorganism and a standard component of human intestinal microbiota. Besides GIT, it is present in the mouth, skin, and vagina. Although *C. albicans* acts as a commensal, sometimes it becomes an opportunistic pathogen. This shift happens when the host is affected by an illness which demands use of antibiotics or if the immune status changes. The result of this dysbiosis is *Candida* overgrowth. The problem of increased occupation is translocation through the intestinal barrier and spreading through the bloodstream (candidaemia) and invading other organs. Infections caused in this way can be very dangerous or lethal. The core of *Candida*'s virulence is the diversity of its cell morphology. Apparently, this yeast can be in unicellular form and two different filamentous forms (pseudohyphae and hyphae) (Huseyin et al., 2017). In order to prevent commensal to pathogen switch, it is vital to control the diet (avoiding the Western diet) and reduce the intake of fungal or bacterial drugs (long-term and repeated use of broad-spectrum antibiotics). Healthy food ensures a healthy microbiome, suppressing the overgrowth of hyphae and abnormal colonisation (Kumamoto et al., 2020). This skin, mucosal, deep-seated organ infections (intra-abdominal abscess, peritonitis) and

osteomyelitis are called candidiasis and can happen at any age. These dangerous infections are widely spread and recognised as a leading cause of mortality in clinical settings. *Candida albicans* is recorded as a primary pathogen, but at least 25 *Candida* spp. cause infections. Besides *C. albicans, Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei* and *Candida auris* are emerging and threatening human health (Pappas et al., 2018). Only four types of antifungal drugs are used to treat candidiasis. Azol drugs inhibit ergosterol synthesis in yeast cell membranes, inhibiting fungal growth. Polyene drugs, namely amphotericin B, bind ergosterol to the lipid bilayer, which destroys yeast's membrane structure. The third category, pyrimidine analogues, blocks DNA synthesis in fungi, while the fourth includes echinocandin, which explicitly interrupts the formation of fungal cell walls. The main disadvantage of these medications is the advancing drug resistance of fungi. These medications prevent fungal growth, but after long-term treatment, imbalance occurs in human flora, leading to compromised immunity which favours fungal infections. Lately, various studies have demonstrated how pharmaceutical probiotics inhibit pathogenic fungi. This is an alternative to traditional therapeutic drugs, which are getting more and more attention (Wu et al., 2021). Most of the data about fungal-bacteria interactions are collected from *in vitro* surveys or non-intestinal mucosal settings. Therefore, it is complex to define a model for good interpretation of these mechanisms (Pérez, 2021). Fungicidal and fungistatic effects of probiotics are mostly evaluated in *in vitro* conditions. Probiotics, primarily lactic acid bacteria (LAB), produce secondary metabolites with antimicrobial activity, compete for nutrients and adhesion sites, stimulate the immune system and suppress *Candida albicans* biofilm formation. On the other hand, some studies confirmed that these bacteria could induce the downregulation of genes associated with biofilm and hypha formation. Still, it is a big question whether this inhibition depends on direct contact between fungi and bacteria, or whether it is a result of the secretion of some metabolites by probiotics (Matsubara et al., 2016).

2.2.1. Filamentation inhibition

The morphological transition from yeast to hyphae formation is considered a key virulence factor of *C. albicans*. Yeast cells take this form to defend themselves from the attack of macrophages. Mycelium probably damages epithelial and endothelial cells by secretion of hydrolytic enzymes, accessing the bloodstream and causing candidemia. Nevertheless, it is possible to influence the induction of hyphal growth by interrupting signal transduction pathways (Sudbery, 2011). In 2004, Noverr and Huffnagle conducted *in vitro* studies with live

Lactobacillus rhamnosus, *Lacobacillus casei* and *Latobacillus paracasei* bacteria and their supernatant. The results showed how short-chain fatty acids, especially butyric acid, can inhibit the germination of *C. albicans*. These acids have an anti-inflammatory function and are produced in large quantities by LAB. Another group, Vilela et al. (2015) found that the probiotic bacterium *Latobacillus acidophilus* inhibits biofilm formation and filamentation of *C. albicans*. The experiment showed a smaller number of hyphae when *C. albicans* and *L. acidophilus* were in interaction. The culture filtrate of *L. acidophilus* also has reduced the number of *C. albicans* hyphae. Scanning electron microscopy (SEM) images revealed reduced *C. albicans* filamentation when the fungus was in touch with live *L. rhamnosus* cells and their supernatant. However, in this case, a decrease in pH value in the environment was not observed, so it is assumed that this bacterium interferes with hypha-specific gene expression and, in that way, suppresses filamentation (Matsubara et al., 2016).

2.2.2. Inhibition of growth and biofilm formation

The growth of *C. albicans* depends on the pH value of the environment, meaning that acid conditions fit yeast form, while alkaline conditions favour hyphae filamentation. Organic acids, mostly lactic acid, produced by *Lactobacilli* decrease pH, leading to inhibition of this fungus. Besides lactic acid, the bacterium *L. reuteri* produces hydrogen peroxide, which also acts as an antifungal compound. Moreover, LAB have a fungicidal effect on yeast cells. Again, lactic acid causes a loss of metabolic activity of *C. albicans* cells and eventually kills them (Köhler et al., 2012).

Biofilm is a formation derived from yeast or bacterial cells to protect them. These organised cell communities are incorporated with an extracellular polymeric matrix and attached to some surfaces. Biofilms hinder the penetration of antifungal drugs and improve the resistance of fungal cells. The biofilm formation consists of three phases, early (adhesion), middle (initial colonisation) and mature phase. In the beginning, yeast cells must adhere to the specific surface with the help of bacteria, forming a microcolony. Then, more microcolonies connect and form the base layer by secreting polymers for matrix formation. Gradually, hyphae and pseudohyphae develop and enlarge biofilm until it reaches the final stage. Drug resistance emerges and evolves after this process, making fungal infections more intractable (Wu et al., 2021). Matsubara et al. (2016) investigated the development of *C. albicans* biofilm throughout these three phases. Moreover, their research included the influence of different LAB (cell suspensions and cell-free supernatants) on that process. The results have shown how *L.*

rhamnosus, L. casei and *L. acidophilus* inhibited both initial colonisation and development of mature biofilm. Besides, this research showed that the density of LAB matters, and for every strain, different cell concentration is needed for inhibition. Furthermore, they found that *L. rhamnosus* supernatant also inhibits biofilm formation and development, but not mature biofilm (less than planktonic LAB cells). According to that, only 24-h and 48-h supernatants contained exometabolites that could obstruct biofilm formation.

2.3. GROWTH INHIBITION OF BACTERIA BY YEASTS

For a long time, scientists have been aware of the importance of symbiosis between the human body and microorganisms. This is how the term 'microbiome' became popular. However, until 2010 it referred only to commensal and pathogenic bacteria. Finally, all other microbes gained importance, especially fungi. Since then, the expression 'mycobiome' has been in use, designed as a combination of the words mycology and microbiome. So, this history explains why this field of research is still fresh (Cui et al., 2013). Another reason is a long ignorance towards the fungi in the human body and their interaction with the host and other microorganisms. As a result, these fungi were co-isolated with pathogenic bacteria and, because of that, underestimated and considered harmless. But, in the last decades, awareness about infections caused by yeasts increased significantly, so the interaction between them and bacteria has become one of the significant issues (Krüger et al., 2019). The aforementioned infections are better explained in the last chapter, where it is shown how probiotic bacteria can help patients by inhibiting the influence of contagious fungi. However, it is rarely explained and studied how yeasts can antagonise bacteria, and inhibit their growth and influence (Richard and Sokol, 2019).

2.3.1. *Saccharomyces cerevisiae* versus *Staphylococcus aureus*

Staphylococcus aureus is a major cause of a wide range of infections. It is an opportunistic, Gram-positive bacterium, human commensal, that colonises the anterior nares of 20-25 % of healthy adults. Secreted exo-enzymes and toxins by planktonic bacterial cells cause a variety of food poisonings and acute infections like bacteremia and skin abscesses. Another important growth form is biofilm. It is hazardous since *S. aureus* can attach to bones or heart valve, which leads to osteomyelitis and endocarditis. Onwards, biofilms invade medical devices and implanted materials such as pacemakers, catheters and prosthetic joints. This form is especially vicious because the biofilm matrix holds back macrophages, enabling the immune defence. As

a result, there are antibiotic resistant biofilms of *S. aureus* (Lister and Horswill, 2014). Again, emerging antibiotic resistance motivated scientists to find some practical and simple alternatives. Many studies have proven how probiotics can improve human health and help in the struggle with pathogenic bacteria. The most significant part of the research about probiotics is dedicated to bacterial probiotics. Recently, yeasts became an interesting substitution. Gastric juices in the stomach have a low pH, ensuring extreme conditions for many microorganisms. Few yeast strains can handle those conditions, among which are *Saccharomyces cerevisiae* strains. Usually, this fungus is known as an industrial microorganism and as a model eukaryotic organism. However, some studies have shown bio-therapeutic properties in the case of diarrhoea and colitis. It is assumed that *S. cerevisiae* owes its antibacterial capability to the secretion of inhibitory proteins and extracellular protease, stimulation of immunoglobulin A, and acquisition or elimination of secreted toxins (Shruthi et al., 2022; Fakruddin et al., 2017). Fakruddin et al. (2017) investigated the antibacterial activity of yeast *S. cerevisiae* isolate (whole cells), its supernatant and lysate on many bacteria, including *S. aureus*. The best inhibition was achieved with cell lysate and then with whole cells. Culture supernatant showed antimicrobial activity, but it was not so significant, implying how this antagonistic relationship is not based on extracellular compounds. Moreover, Saidi et al. (2019) took another step forward and examined the influence of *S. cerevisiae* on both methicillin-sensitive (MSSA) and resistant (MRSA) *S. aureus*. In this case, only the supernatant extract had antibacterial activity against MRSA and MSSA. However, bactericidic and bacteriostatic effects are not the only consequences of antibacterial agents. It is vital to discover virulence factors of pathogenic bacteria and find a way to inhibit them. *S. aureus* has three important virulence factors, including biofilm, α-hemolysin and enterotoxin A. Supernatant and lysate extracts of *S. cerevisiae* lessen biofilm formation of MSSA and MRSA. In addition, the supernatant extract inhibited the expression of α-hemolysin and enterotoxin A genes of MRSA and MSSA strains. Another team (Kim et al., 2020) explored the influence of the cell-free supernatant of *S. cerevisiae* on this bacterium. Their results showed significant inhibition of biofilm formation and degradation of a mature biofilm. Namely, compounds in the cell-free supernatant of *S. cerevisiae* can modify gene expression related to biofilm formation. Adhesion ability, autoaggregation ability and production of exopolysaccharide are linked with bacterial attachment and biofilm formation. Again, supernatant managed to decrease these indicators, showing how the fungus *S. cerevisiae* can be effective prevention of *S. aureus* infections.

2.3.2. *Kluyveromyces marxianus* versus *Vibrio cholerae*

Cholera is one of the most frequent epidemic waterborne diseases in developing countries, where outbreaks happen regularly. It is caused by the motile, Gram-negative curved rod bacterium *Vibrio cholerae.* The main symptom is watery diarrhoea leading to dehydration, resulting in the death of 50-70 % of untreated patients. After ingested, the pathogen *Vibrio cholerae* colonises the small intestine and excretes an enterotoxin known as cholerae toxin (Faruque et al., 1998). *Kluyveromyces marxianus* yeast is a sister species to *Kluyveromyces lactis*. It is a homothallic, hemiascomycetous fungus recognised by its capacity to assimilate lactose and use it as a carbon source. So, it makes sense that it is often isolated from dairy products such as cheese, yoghurt, or fermented milk. Just like *Saccharomyces cerevisiae* or LAB it has GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) status. In addition, this fungus meets the criteria to be specified as probiotic - it can be produced on an industrial scale, and it can tolerate the gut environment while effects on host's welfare (Homayouni-Rad et al., 2020; Lane and Morrissey, 2010). Interaction among these two microbes is characterised as antagonistic, and it is recognised due to the examination of kefir secreted molecules and pathogenic microorganisms. 70 % of present genera in kefir were *Kluyveromyces marxianus,* and others were mostly *Lactobacillus* species. More precisely, this antagonistic relationship is based on a specific compound produced by *K. marxianus,* tryptophol acetate. This product disrupts quorum sensing (QS) signal cascades of *V. cholerae* and consequently affects many virulence factors (Malka et al., 2021). QS implies cell-cell communication. It is a ubiquitous occurrence in bacteria, which harmonises gene expression. They send each other many ″messages″, but in this case, it is important to mention the regulation of genes that promote the invasion, defence, and spread of these pathogens. Communication occurs due to secreted QS signalling molecules, similar to human hormones. So, it is supposed that blocking these signals can hinder the production of virulence factors (e.g., toxins, proteases, immune-evasive factors) and biofilm formation, which is a root for antimicrobial resistance. If the communication of bacteria were blocked, they would live as individuals, making it impossible to promote pathogenesis and harm the host (LaSarre and Federle, 2013). Tryptophol acetate, a metabolite, does not affect the growth of *V. cholerae* but its biofilm by direct inhibition of QS pathways. This is very important because enhanced biofilm generation enables increased secretion of cholerae toxin. To be accurate, tryptophol acetate represses the transcription of *hapR* and *ctxA* genes. Repression of *hapR* leads to transcription inactivation of gene *hapA,* which is necessary for the expression of proteases. Moreover, tryptophol acetate

directly represses transcription of the gene *ctxA*, which codes for chloreae toxin. Overall, metabolite secreted by *K. marxianus* can very efficiently inhibit this virulence system, where biofilm generation relates to the virulence of *V. cholerae*, especially with the secretion of cholerae toxin (Malka et al., 2021).

2.3.3. *Candida albicans* versus *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogenic Gram-negative soil bacterium. It is a rod-shaped, asporogenous, and monoflagellated microorganism which favours a temperature range between 25 °C and 37 °C. Adaptability to many complex growth environments ensures the colonisation of various plants, animals, and humans, which causes infections, mainly in immunocompromised patients. The most important product of *P. aeruginosa* metabolism is the blue-green pigment pyocyanin. However, this metabolite is not only a pigment but also the virulence factor of *P. aeruginosa*. It interferes with cell respiration, epidermal cell growth, ciliary beating, calcium homeostasis and prostacyclin release from lung endothelial cells. Those infections were treated with traditional antibiotics, which inhibit essential bacterial functions and, in that way, inhibit the growth or kill pathogens. However, this mechanism of action foists selection pressure resulting in resistant bacterial strains. Therefore, the proper agent would not affect the growth of bacteria (it is linked with the probability of resistance). The direct pressure on virulence factors leaves bacteria defenceless and enables the immune system to attack *P. aeruginosa*. The development of antibacterial approaches has helped to discover many natural or synthetic compounds that can inhibit virulence factors (Wu et al., 2015). Farnesol is the first quorum-sensing molecule identified in eukaryotes. Together with tyrosol and farnesoic acid, it makes three autoregulatory compounds produced by the fungus *Candida albicans*. *C. albicans* uses this volatile sesquiterpene to control the transition from yeast to filamentous growth form. Not only does farnesol affect conditions of *C. albicans*, it also regulates signals of *P. aerugionosa*, the permeability of *S. aureus* or *E .coli*. Moreover, it induces apoptosis in *Aspergillus nidulans* and *Aspergillus flavus* (Leonhardt et al., 2015). *C. albicans* secretes farnesol in conditions when it is necessary to send a message that the population is dense enough and the hyphal growth should be stopped. When there is enough quantity of farnesol, it can affect *P. aeruginosa*. To be precise, farnesol decreases the production of *Pseudomonas* quinolone signal (PQS) and the PQS-controlled virulence factor, pyocyanin. PQS is secreted compound (2-heptyl-4-quinolone) that participates in QS of *P. aeruginosa* and controls the expression of genes. Together with the transcription factor, PqsR, PQS regulates the production

of hydrogen cyanide, extracellular proteases and redox-active phenazines, including pyocyanin. It directly affects transcription of *pqsA*, the first gene in PQS biosynthetic operon. The best signal disruption occurs at the beginning when the PQS concentrations are low. Thus, co-cultures of *C. albicans* and *P. aeruginosa* have reduced concentrations of PQS and pyocyanin. It corresponds to the survival of *C. albicans*, otherwise sensible to the toxicity of this pigment. Mixed interactions of these two microorganisms are common and present in different forms. So, not only does fungus negatively affect the bacterium, but it is also possible to be vice versa. *P. aeruginosa* forms biofilm on the top of fungal hyphae, affects *C. albicans* morphology and kills its cells by secreted phospholipases and phenazines (Cucgini et al., 2007).

2.4. BACTERIA AS PROBIOTICS

″Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host″ (Hill et al., 2014). This is why they are added to many functional foods and dietary supplements. To be called probiotics, these microorganisms should meet certain criteria:

1. Beneficial effect on the host.
2. Non-pathogenic, non-toxic and free of significant adverse side effects.
3. Able to survive through the GIT.
4. The adequate number of viable cells in the product.
5. Compatibility with the product matrix, processing, and storage conditions so that the desired properties can be maintained and labelled accurately (Fontana et al., 2013).

The use of probiotics is extensive, but in general, they support the well-being of gut microbiota. Probiotics can ease illnesses like diarrhoea, Crohn's disease, irritable bowel syndrome, *Helicobacter pylori* infections, and ulcerative colitis. The other welfares of taking probiotics are the prevention of colon cancer, alleviation of lactose intolerance, reduction of food allergies, lowering of serum cholesterol and playing an important role in the modulation of the immune system. This term represents a mono culture or mixed culture of live microorganisms. The most recently used microorganisms are *Lactobacillus* and *Bifidobacterium* species, but there are others, like *Bacillus* sp. and *Enterococcus* sp. (Williams, 2010). The probiotic effect on health is strain-specific, meaning that different bacteria result in dissimilar welfare. However, it can be said that one of the essential properties of probiotics is protection against pathogens in the

intestinal tract of the host. The mechanisms that ensure that effect are a modification of gut microflora, competitive adherence to the mucosa and the epithelium, strengthening of the gut epithelial barrier, and finally, modulation of the host's immune system. So, maybe it is best to use a mixed culture because it ensures a broader range of beneficial results (Fontana et al., 2013).

2.4.1. *Lactobacillus acidophilus*

Lactobacilli are the most prominent probiotic bacterial species recognised in various dairy products and the human GIT. Generally, they are Gram-positive, non-motile, non-sporulating, round or rod shape bacteria. *L. acidophilus* uses glycolysis or EMP pathway to ferment hexoses and produce D and L- lactic acid. This thermophilic strain prefers temperatures between 30 and 45 °C, and pH 4-5. It is mainly isolated from fermented products, especially yoghurt, but also from numerous food products, e.g., fruits, milk, meat, vegetables, and cereals, where this bacterium is indigenously present. Besides its benefits as a starter culture for many fermented processes, *L. acidophilus* is recognised as a producer of promising agents. They are known as bacteriocins, antimicrobial peptides produced by strains of certain species that show antimicrobial properties to closely related bacteria and some pathogens. *L. acidophilus*-produced bacteriocins are heat-stable and non-lantibiotic small peptides. Their addition to many food products provides preservation and contribution to a specific taste, flavour and texture. Ingested *L. acidophilus* bacteria colonise the human GIT and protect it from pathogens (Anjum et al., 2014).

2.4.2. *Bacillus clausii*

Bacillus spp. are Gram-positive, rod-shaped, spore-forming, aerobic or facultative anaerobic bacteria. These strains are omnipresent and can be found in the air, water, soil, all kinds of fermented food and raw vegetables, and human and animal GIT. Despite this fact, it is considered that these bacteria colonise gut microbiota only after intake of food contaminated by soil microorganisms. In nature, they are mostly present in form of spores, enabling them to survive in extreme conditions. This is why these spores live in the stomach, very acid environment and in the GIT where they tolerate bile salts and other unfriendly products. The primary action mechanism of these bacteria is the modulation of gut microbiota composition by producing metabolites that act antimicrobial, like bacteriocins and antibiotics. Also, they excrete some valuable products, including vitamins, peptides, and extracellular enzymes. That

results in the growth of desirable microbes, while the growth of those which harm the host's health is suppressed (Elshagabbe et al., 2019).

Along with the *Lactobacillus* species, they are considered the most efficient probiotic bacteria. However, the main difference is that *Bacillus* sp. form spores. It makes them more stable and viable, mainly because of tolerating high or low temperatures, making them easier to store. Also, there is a problem of low pH values in the stomach that are not an issue for the *Bacillus*, as can be for the *Lactobacillus* (Cutting, 2011). Consumption of antibiotics often creates imbalance in gut microbiota and leads to diarrhoea and chronic colitis. *Bacillus clausii* strains are an especially desirable choice for this disorder because of their resistance to several types of antibiotics. The vast spectrum implies penicillins, cephalosporins, aminoglycosides, macrolides, tetracycline, chloramphenicol, and rifampicin. Also, these bacteria are prevalent for another reason - production of lantibiotics, ribosomally synthesised, post-translationally modified peptides. Lantibiotics inhibit Gram-positive bacteria by interruption of peptidoglycan biosynthesis. Moreover, *B. clausii* has immune-modulatory properties like alleviating nasal symptoms during allergic reactions, anti-inflammatory effects against the side effects of antibiotic-based *Helicobacter pylori* therapy, and therapeutic effects against urinary tract infection. Also, it can modify the expression of some genes associated with inflammatory, immune and defence responses, intestinal permeability, cell adhesion, growth, differentiation, signalling, apoptosis, transcription and signal transduction (Lee et al., 2019; Lopetuso et al., 2016).

2.5. YEASTS AS PROBIOTICS

Probiotics are a natural part of human microbiota and can be autochthonous or allochthonous. However, this name mainly alludes to bacteria, especially LAB and *Bifidobacterium* sp. Lately, yeasts have been recognised as an excellent alternative to bacterial probiotics because of their natural resistance to antibacterial antibiotics and the impossibility of resistant-gene transfer to pathogenic bacteria. However, only recent research revealed new and interesting species besides *Saccharomyces cerevisiae* and *S. cerevisiae var. boulardii*. They are mostly found in fermented food and include *Debaryomyces*, *Pichia*, *Torulaspora*, *Kluyveromyces*, *Hanseniaspora*, *Rhodotorula*, *Candida*, *Williopsis* and *Wickerhamomyces* (Shruthi et al., 2022; Lara-Hidalgo et al., 2017). *Saccharomyces boulardii* CNCM I-745 belongs to *Sacharomyces cerevisiae* species, and it is the first probiotic yeast isolated from nature. French microbiologist Henri Boulard has recognised its influence on people with diarrhoea and separated it from

tropical fruit peels. It improves the digestive capacity and helps cure and prevent various diseases, mostly diarrhoea, but also *Helicobacter pylori* infections, inflammatory bowel diseases, irritable bowel syndrome, candidiasis, and dyslipidemia. This strain shares more than 99 % genomic relatedness with *S. cerevisiae* strains that are not characterised as probiotics. The main difference is the higher production of acetic acid, which is one of the mechanisms of probiotics against pathogenic bacteria (Kaźmierczak-Siedlecka et al., 2020).

Debaryomyces hansenii is a haploid yeast whose reproduction is of vegetative nature through multilateral budding. It is found in hypersaline waters because it is osmo-, halo- and xerotolerant fungus. Except for salt water, it is isolated from wine, beer, fruit, cheese, meat, and soil. *D. hansenii* strains are very heterogeneous and versatile, making them suitable for fermentation of many carbon sources, growth in different conditions and expression of various protease and lipase activities. The temperature range suitable for their growth is wide as well. The optimal temperature is at 20-25 °C, but sometimes it is possible for it to grow in the range of 5-10 °C, or even below 0 °C. *D. hansenii* is non-fermentative yeast, meaning that it can not grow in environments with low oxygen presence. This fungus has significant biotechnology potential because of its ability to accumulate lipids (up to 70 % of its dry biomass) (Breuer and Harms, 2006). Moreover, *D. hansenii* demonstrates some desirable features by producing certain valuable compounds. As mentioned above, probiotics must be able to survive hostile GIT conditions. This extremophilic yeast endures harsh conditions, low pH and high bile salt concentrations. Another criterion is the ability of yeast cells to aggregate and adhere to abdominal epithelial cells. *D. hansenii* cell surfaces are highly hydrophobic, which ensures good aggregation and adhesion. Furthermore, *D. hansenii* produces killer toxins that can inhibit the growth of many fungal pathogens (e.g. *Rhizopus stolonifera*, *Aspergillus niger*, *Alternaria brassicola* and *Alternaria citri*) (Shruthi et al., 2022).

3. EXPERIMENTAL PART

The following experiments were carried out at the Department of Food Microbiology and Biotechnology of Faculty of Food Science at the Hungarian University of Agriculture and Life Science in the frame of a CEEPUS scholarship.

3.1. MATERIALS

3.1.1. Samples used for isolation of yeasts and bacteria

The samples used to isolate yeasts were pear (with some rotten spots), soft cheese and yoghurt. All of them were purchased from the local supermarket in Budapest, Hungary.

Isolation of probiotic bacteria was conducted from two samples. Both of them are commercial probiotic supplements that can be found in pharmacy stores in Budapest, Hungary. LAB were isolated from the Protexin Junior +C tablet (Manufacturer: ADM Protexin Limited, Lopen Head, Somerset, TA135JH, United Kingdom), and *Bacillus clausii* from the Normaflore suspension (Manufacturer: Laboratoire Unither, Zl de la Guérie, FR-50211 Coutances France; Sanofi SpA, Viale Europa 11, IT-21040 Origgio Italy). Table 2. represents the composition of the probiotic supplement Protexin Junior +C.

Table 2. List of probiotic bacteria present in Protexin Junior +C which was used for isolation of LAB

PROBIOTIC BACTERIA CONTAINED IN PROTEXIN JUNIOR +C
Lactobacillus paracasei PXN ® 37 ™
Lactobacillus rhamnosus PXN ® 54 ™
Streptococcus thermophilus PXN ® 66 ™
Bifidobacterium breve PXN ® 25 ™
Lactobacillus acidophilus PXN ® 35 ™
Bifidobacterium infantis PXN ® 27 ™
Lactobacillus bulgaricus PXN ® 39 ™

3.1.2. Media (agars and broths)

Yeast Extract Peptone Dextrose agar (YEPD) is a complete medium for yeast growth. It was used for the isolation and maintenance of yeast cultures. Yeasts were kept on YEPD plates and slants, and used for all experiments. The media consisted of 0.5 % peptone, 0.5 % yeast extract, 1 % glucose and 1.5 % agar. In the case of isolation of yeasts from the samples, chloramphenicol (0.1 g/L) was added to YEPD to prevent the growth of many bacteria.

Bismuth Sulfite Agar (BSA) is a selective and differential medium. Its selectivity is based on the presence of inhibitors, while it is differential due to hydrogen sulfide production. Bismuth sulfite and brilliant green inhibit the growth of Gram-positive bacteria. It was used for isolating mainly *C. albicans*, other *Candida* species and further genera of yeasts from the samples, and for the preparation of pure cultures. In the case of isolation from the samples, chloramphenicol (0.1 g/L) was also added. Onwards, this agar was also used in the experiment where growth of the yeasts was compared on different media. BSA was a previously prepared medium of MERCK (Merck KGaA, 64271 Darmstadt Germany).

De Man, Rogosa and Sharpe (MRS) broth (Lab M; Neogen company, Lancashire United Kingdom) is a liquid medium used to cultivate and enumerate LAB. It was used for the isolation of bacteria from the samples (formulated tablets), storage of LAB, contact inhibition test, and test of the inhibitory effect of cell-free cultivation media.

MRS agar is a selective culture medium designed to favour the growth of *Lactobacillus* sp. It was used for the maintenance of LAB, contact inhibition test and test of the inhibitory effect of cell-free cultivation media. It was prepared by adding 1.5 % agar to the previously described MRS broth.

Modified MRS (mMRS) is a differential medium for lactic acid bacteria. It is appropriate for counting and differentiating each LAB present in mixed culture. It was prepared by adding 1.5 % agar, 0.05 % L-cysteine and 0.002 % bromophenol blue to the MRS agar.

Walerstein Laboratory (WL) medium is used to cultivate yeasts, moulds, and bacteria in brewing and industrial fermentations. In this case, it was applied for making pure yeast cultures and their distinction. It contained 4 g/L yeast extract, 5 g/L peptone, 50 g/L glucose, 0.55 g/L KH_2PO_4, 0.425 g/L KCl, 0.125 g/L $CaCl_2$, 0.125 $MgSO_4$, 0.0025 g/L $FeCl_3$, 0.0025 g/L $MnSO_4$, 0.022 g/L bromocresol green, and 15 g/L agar.

Dichloran Rose Bengal Chloramphenicol (DRBC) agar is designed to suppress the growth of spreading moulds and bacteria while supporting yeast growth. It was used for the isolation of yeast colonies contaminated with moulds. This medium was purchased from MERCK (Merck KGaA, 64271 Darmstadt Germany).

Bacillus clausii was isolated on Mueller Hinton Agar (MHA) (Merck KGaA, 64271 Darmstadt Germany). MHA is a non-selective, non-differential medium, a loose agar which absorbs antibiotics and toxins with the help of starch.

Tryptic Soy Broth (TSB) (Biolab Diagnostic Laboratory, Budapest, Hungary) is a complex, general-purpose liquid medium used to grow bacteria with high nutritional requirements. It was used to cultivate *B. clausii* and yeasts for the contact inhibition test, and in examining the inhibitory effect of cell-free cultivation media. Subculturing of *B.clausii* and yeasts B5 and B10 was also conducted in TSB.

Tryptic Soy Agar (TSA) is a general-purpose non-selective growth medium that supports the growth of many bacteria, yeasts and moulds. TSA plates were used for subculturing and maintenance of *B.clausii*, contact inhibition test, the test of the inhibitory effect of cell-free cultivation media, and in the experiment where *B. clausii* was co-cultured with yeasts B5 and B10.

For subculturing experiment adding the antifungal drug nystatin in TSA was needed. The concentration of nystatin in TSA was 0.1 mg/mL.

Rose Bengal Chloramphenicol (RBC) agar (Biolab Diagnostic Laboratory, Budapest, Hungary) is a selective medium for enumerating yeasts and moulds. It was used in the co-culturing test because of its ability to prevent the growth of *B. clausii*.

Potato Dextrose Agar (PDA) (Biolab Diagnostic Laboratory, Budapest, Hungary) is a selective medium for detecting, isolating, and enumerating yeasts and moulds. It comprises dehydrated potato infusion and dextrose that encourage luxuriant fungal growth. PDA was used in the experiment where the cultivation of the yeasts was compared on different media.

Yeast & Mould Agar (Lab M; Neogen company, Lancashire United Kingdom) is a medium recommended for isolating and maintaining yeasts and moulds, and detecting wild yeasts in beer. The medium consists of an enzymatic digest of gelatin, yeast extract, malt extract, dextrose, and agar. Yeast & Mould Agar was also applied to compare yeast growth on different media.

Oxytetracycline Glucose Yeast (OGY) agar-base (Merck KGaA, 64271 Darmstadt Germany) is a selective medium for the enumeration of moulds and yeasts in foods. OGY comprises yeast extract, glucose, biotin, oxytetracycline and agar. This agar was also used for comparison of yeast growth.

Oxytetracycline Glucose Yeast Extract agar (O.G.Y.E.) is a selective medium for isolating and enumerating yeasts and moulds. O.G.Y.E. consists of yeast extract, dextrose, biotin, and agar. The manufacturer was Lab M (Neogen company, Lancashire United Kingdom) and was used to compare yeast growth on different media.

Malt extract agar (Merck KGaA, 64271 Darmstadt Germany) is used for the cultivation of fungi. It is very high in carbohydrates and highly acidic, making it the perfect environment for yeasts and moulds while inhibiting the growth of bacteria. It was also used for the examination of yeast growth on different media.

Nutrient agar (Merck KGaA, 64271 Darmstadt Germany) is a general-purpose nutrient medium used to cultivate microbes, supporting the growth of a wide range of non-fastidious organisms. This agar is very popular as it contains many nutrients suitable for many types of bacteria and fungi. The agar was used to compare yeast growth on different media.

Standard I nutrient agar (Merck KGaA, 64271 Darmstadt Germany) is used for the cultivation of fastidious bacteria. This medium is an excellent base for adding blood, ascites fluid or serum. It was used for the experiment where the growth of yeasts was compared.

TSA was used in two more forms. Firstly, 2.5 mg/L of $FeCl_3$ was added to the medium. Secondly, 0.3 g/L of $Fe(SO_4)_3$. Both media were used for the comparison of yeast growth.

Organic pears puree (Ella's kitchen, Organic pears puree baby pouch 4+ months 70 g, produced in Austria, Wien) was used for the preparation of medium used in the experiment where the growth of yeasts on different media was compared. The following recipe was used in the preparation of three types of media with three different concentrations:

1. 40 mL of puree + 40 mL of 3 % agar (marked as "Pear 2x")
2. 20 mL of puree + 20 mL of distilled water + 40 mL of 3 % agar (marked as "Pear 4x")
3. 8 mL of puree + 32 mL of distilled water + 40 mL of 3 % agar (marked as “Pear 10x”)

3.1.3. Solutions

Dilution liquid was used for the isolation of microorganisms from the samples. On 1 L of distilled water was added 1 g of peptone and 8.5 g of NaCl.

The Breaking buffer (B buffer or lysis buffer) is a solution used to break (open) cells for molecular biology experiments, mainly DNA extraction. It consists of buffering salt (10 mM Tris-HCl) and ionic salt (100 mM NaCl) to regulate the pH and osmolarity of the lysate.

Phenol : Chloroform : Isoamyl Alcohol (PCIA, 25:24:1) was used to separate nucleic acids from proteins and lipids.

TE buffer was used to solubilise DNA or RNA, while protecting it from degradation. A typical recipe for making this buffer is 10 mM Tris and 1 mM EDTA.

RNase (10 mg/mL) was added for cleaving the cellular RNA, which was not required for the cells, during the isolation of the genomic DNA.

TBE buffer (Tris/Borate/EDTA) is a buffer solution containing a mixture of Tris base, boric acid and EDTA. To prepare the 0.5x TBE buffer the previously prepared 10x TBE buffer (108 g/L Tris base, 55 g/L boric acid and 7.5 g/L Na_2EDTA) was diluted: 50 ml of 10x TBE buffer was measured and diluted with 950 ml of distilled water.

DNA dye (GR Green DNA dye, 10000x, Lab Supply Mall; Biocenter Kft, Hungary) was used for gel electrophoresis, which helped the extracted DNA bands to be seen under UV transilluminator.

Loading dye (Blue, 6x, BioLabs Inc, B6021S; Bromophenol blue based dye which consists of SDS and EDTA) was added to the DNA samples in the agarose gel electrophoresis to help the samples sink in the wells.

3.1.4. Instruments

Vortex V-1 plus (Biosan, Riga, Latvia) was used for gentle mixing and resuspension of cells and various samples in tubes.

For the determination of cell concentration, DEN-1B Densitometer was applied (Biosan, Riga, Latvia).

MALDI-TOF MS was used to identify the microorganisms from the sample pure colonies isolated on MRS and YEPD plates. The instrument was Bruker's MALDI Biotyper.

The DNA concentration was measured spectrophotometrically using NanoDrop1000 (Thermo Scientific).

PCR was carried out using Swift maxi thermal cycler (ESCO). The primers NL1 and NL4 were used to analyse the 28S rDNA of the yeast isolates.

The amplified DNA segments were purified using the Monarch PCR and DNA Cleanup Kit (New England BioLabs) and sequenced by BIOMI Ltd. (Gödöllő, Hungary). For sequencing, the NL1 primer was used.

The sequence data were analysed by Mega11 software, and the standard nucleotide BLAST (National Center for Biotechnology Information) was used for identification.

3.2. METHODS

3.2.1. Isolation of yeasts and bacteria

For the isolation of yeasts, three samples were used:

1. Pear

   12.56 g of pear was mixed with 113.04 mL of dilution liquid in the lateral filter bag. Onwards, it was put into the bag mixer for 2 minutes. From that homogenised mixture, three decimal dilutions were prepared. 100 µL of each was smeared on YEPD and BSA plates (both contained chloramphenicol). Petri dishes were put in the incubator at 25 °C.
2. Soft cheese

   For this sample, the procedure was similar. 9.55 g of cheese was put in the lateral filter bag. The next step was the addition of 85.95 mL of dilution liquid and homogenisation for two minutes. 100 µL of the homogenised mixture was directly taken from the bag and smeared on YEPD and BSA plates (both media contained chloramphenicol).
3. Yoghurt

   1 mL of yoghurt was combined with 9 mL of dilution liquid. Again, the content of the lateral filter bag was homogenised for 2 minutes. 100 µL was smeared on YEPD and BSA plates (both contained chloramphenicol).

After the incubation period, 16 different colonies were recognised on BSA and YEPD plates. They were transferred by a streaking method on fresh agar dishes to get pure cultures. Five

were put on DRBC plates because of mould contamination on the original plate, and 11 were put again on BSA. Later, plates with isolated colonies were photographed.

In the meantime, YEPD agar slants were prepared, and all 16 yeast isolates were inoculated onto them. Tubes with the agar slant were incubated at 25 °C for 48 h and then stored at 4 °C.

Two samples were used for the isolation of probiotic bacteria:

1. Protexin Junior +C probiotic supplement
   One tablet was dissolved in 20 mL MRS broth and incubated at semi-anaerobic conditions at 37 °C. After 48 h loopful of the sample was streaked on two mMRS plates. Plates were stored under the same conditions. After incubation, 4 different colonies were recognised. Loopful of those cells was inoculated in MRS broth tubes. After 24 h of incubation, those 4 isolates (A, B, C, D) were spread on MRS plates.

2. Normaflore suspension
   Loopful of suspension was taken from the container and spread on MHA. It was incubated at 37 °C for 48 h and then stored at 4 °C.

3.2.2. Comparation of yeast growth on different media

In order to determine the best growth media for each yeast isolate, 15 different media were tested. Three media were prepared from pear-puree, while the other twelve were the following: BSA, PDA, Yeast & Mould agar, OGY-agar, O.G.Y.E., Malt extract agar, Nutrient agar, Standard I nutrient agar, TSA, TSA with addition of $FeCl_3$, TSA with added $Fe(SO_4)_3$ and YEPD. 48-h-old yeast cultures were inoculated on each medium. Petri dishes were incubated, and results were evaluated and photographed.

3.2.3. Properties of the yeast colonies and cell morphology

After the procedure described in chapter 3.2.1, sixteen different yeast cultures were isolated. Each was taken with the microbiology loop and streaked on WL plates to get separated colonies. Those plates were incubated at 25 °C, and after seven days, photos were taken.

The morphology of the yeast cells was examined by a light microscope (at 400x magnification). They were described based on four key characteristics (cell shape, cell division pattern, cell clustering pattern and vacuolation).

3.2.4. Identification of the isolates by MALDI-TOF MS

Four isolates from Protexin Junior +C and 16 yeasts were subjected to the identification. LAB were inoculated on MRS plates and incubated at semi-anaerobic conditions (37 °C) for 24-48 h. Yeasts were inoculated on YEPD plates and incubated at 25 °C for 24-48 h. Loopful of the pure cultures was placed onto steel target plates and air dried. Procedure:

1. 1 µL 70 % formic acid was added to the samples for breaking cell walls
2. Samples were overlaid with 1 µL of HCCA (α-cyano-4-hydroxy-cinnamic acid) solution
3. Air dried at room temperature and analysed by the Bruker MALDI Biotyper System

3.2.5. Contact inhibition test

3.2.5.1. Determination of medium and conditions suitable for co-culturing of yeasts and probiotic bacteria

In order to conduct a contact inhibition test, it was necessary to find a medium where bacteria and yeasts could grow as well. YEPD was already known as a good option for yeasts, and MRS for LAB. The third examined medium was TSA, known as a general-purpose medium.

24 h old culture of the chosen yeast (B10) was taken with the loop and put in 2 mL of distilled water. The suspension was vortexed, and the cells were counted in Bürker chamber. Ten times serial dilutions were prepared, and aliquotes of 100 µL of 100 CFU/mL suspension were spread onto the plates. In the case of LAB, one of the isolates (D) was chosen. Optical density (OD) (λ=550 nm) was set to 0.5 (approximately 10^8 CFU/mL). Again, serial dilutions were prepared, and 100 µL of 100 CFU/mL suspension were smeared on the plates. An identical procedure, as in the case of LAB, was used for *B. clausii.* All three microorganisms were inoculated on YEPD, MRS and TSA plates in two parallels. Half of the plates were put in the incubator at 30 °C, while the others were placed at the same temperature under semi-anaerobic conditions.

3.2.5.2. Inhibition of probiotic bacteria by the yeasts

For this experiment, four LAB isolates were inoculated in MRS broth and kept in the incubator for 48 h (37 °C, semi-anaerobic). *B. clausii* was inoculated in TSB and incubated for 48 h at 37 °C. Yeast strains were inoculated on YEPD plates and incubated at 25 °C for the same time period.

1. The procedure for each LAB was the same and done in two parallels. The broth was poured out of the tube, and the bacterium cells were left at the bottom. 2 mL of distilled water was added and then vortexed. Onwards, 1 mL of cell suspension was put on MRS plate. The Petri dish was rotated until the whole surface was covered with suspension. The leftovers of suspension were taken out with a pipette, and the plate was left to air-dry. Afterwards, thin lines of 48-h old yeast cultures were placed on the MRS plate with the sterile toothpicks.
2. *B. clausii* cells were at the bottom of the tube, so they were easily separated from the TSB. After the broth was spilt, 2 mL of distilled water was added. In another tube, optical density (λ=550 nm) was set at 0.5, and 1 mL of prepared suspension was put on TSA plates. The yeast isolates were streaked on these plates in the same way as described above.

All Petri dishes were put in the incubator at 30 °C. After 48 h, results were evaluated and photographed.

3.2.5.3. Inhibition of yeasts by the probiotic bacteria

Sixteen yeast strains were inoculated on YEPD plates and incubated at 25 °C for 48 h. LAB were inoculated on MRS plate and kept in incubator for 48 h (37 °C, semi-anaerobic). *B. clausii* was inoculated on TSA plate and incubated for 48 h at 37 °C. 48-h-old yeast cells were taken with the loop and suspended in distilled water. Concentration was set by optical densitometer on OD (λ=550 nm) ≈ 0.5. 1 mL of suspension was pipetted on the TSA plate and thoroughly rotated until every part of the medium was covered. After that, it was necessary to take out the leftovers of suspension and let the plate air-dry. Each 48-h-old bacterium was scratched applying toothpicks on the yeast-inoculated TSA. This procedure was done by all of the yeast isolates.

The inoculated Petri dishes were put at 30 °C for incubation. After 72 h, the results were analysed and photographed.

3.2.6. Inhibitory effect of cell-free cultivation media

This experiment was conducted with three yeasts (D4, B5 and B10) and two bacteria (LAB C and *B. clausii*). Yeasts were inoculated in MRS broth and TSB, while LAB C and *B. clausii* was inoculated into MRS broth and TSB, respectively. Incubation lasted 48 h, for yeasts at 25 °C, and for bacteria at 37 °C. After incubation, 1 mL of broth (all samples) was transferred to

Eppendorf tube and centrifuged for 5 min at 12000 rpm and 4 °C. Cell-free cultivation media was collected and filtered through a sterile syringe micro-filter (PES) of 0.45 µm pore size (FilterBio, Labex Ltd, Hungary).

The first part of the well diffusion test was conducted as follows in two parallels. One MRS and one TSA plates were overlaid with an overnight LAB culture (about 10^8 CFU/mL). After it was dried, three wells (7 mm in diameter) were formed and filled with the yeast cultivation media. In the case of MRS agar plate, the growth media were taken from the yeasts propagated into MRS broth, while in the case of TSA plate, the cultivation media were derived from yeasts incubated in TSB. For *B. clausii*, only TSA plate and TSB yeast growth media were used.

In the second part, TSA and MRS agar plates were overlaid by the yeasts. On each plate, two wells were formed and filled with LAB and *B. clausii* cultivation media.

All Petri dishes were incubated at 30 °C for 48 h. The antagonistic effect resulting in the appearance of the clear zones was inspected.

3.2.7. Co-culturing test

In this experiment, the interaction between *B. clausii* and yeasts B5 and B10 was examined. 24-h-old cultures of *B. clausii* and yeasts B5 and B10 were prepared, and 10^8 CFU/mL concentrations were set. 1 mL of prepared suspensions was transferred to a flask containing 100 mL of TSB. Each microorganism was inoculated into a separate flask. Co-culturing was proceeded into two additional TSB containing flasks, as follows:

1. *B. clausii* + B5 (inoculated by 1 mL of suspension from *B. clausii* flask + 1 mL of suspension from B5 flask)

2. *B. clausii* + B10 (inoculated by 1 mL of suspension from *B. clausii* flask + 1 mL of suspension from B10 flask)

Time 0 (determination of cell concentration):

From all five flasks dilution series were prepared, and 100 µL of suspensions with dilution factors 10^2, 10^3, and 10^4 were inoculated on the plates and incubated at 30 °C. In addition, samples from the *B. clausii*, B5 and B10 flasks were spread on TSA plates, and those from the co-culturing flasks on TSA containing nystatin and RBC agar plates. Onwards, flasks were thoroughly shaken and put into an incubator with the inoculated Petri dishes at 30 °C.

After 48 h:

Dilution series were prepared once more and inoculated on the same agar plates as before. Then, again, they were incubated at the same conditions.

Colonies were counted, and results were evaluated.

3.2.8. Sequence analysis of two yeast isolates

3.2.8.1. Extraction of genomic DNA

For extraction of genomic DNA from the pure yeast colonies, overnight cultures were prepared and used. First, 1 mL sterile distilled water was pipetted into the Eppendorf tubes, and then a loopfull of cells was added into the water and mixed by vortexing for 10 seconds. Then the tubes were centrifuged at maximum speed (14000 rpm) at 4 ℃ for 5 minutes. The supernatant was poured out, and 200 µL of B-buffer and approximately 0.3 g of glass beads were added. Afterwards, 100 µL of phenol solution and 100 µL of chloroform-isoamyl alcohol were added, and vortexed for 3 minutes. Later, 200 µL of TE buffer was added and vortexed briefly. After this step, the samples were centrifuged for 10 minutes at maximum speed. Then, the upper phase (approximately 400 µL) was pipetted into another new Eppendorf tube containing 800 µL of 96 % ice-cold ethanol and mixed by inversion. The samples were then put in the freezer (-20 °C) for 10 minutes. Next, they were centrifuged as before, and the supernatant was discarded. 50 µL of TE buffer and 2 µL of RNase (50 mg/mL stock solution) were added to the pellet and incubated for 30 minutes at 60 ℃. After incubation, 100 µL 96 % ice-cold ethanol was added to the sample, mixed by inversion, and centrifuged as before. The supernatant was discarded, and after drying the pellet, 50 µL of TE buffer was added to the tubes and stored in the freezer (-20 °C).

The DNA concentration was measured spectrophotometrically using NanoDrop1000 (Thermo Scientific) and adjusted to 50 ng/µl for specific amplification of D1/D2 variable domain of the large subunit of rRNA.

3.2.8.2. PCR amplification

The primers NL1 (5’-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5’-GGTCCGTGTTTCAAGACGG) were used for the analysis of 28S rDNA of the yeast isolates, and the following protocol was used for the PCR: (i) 95 ℃ for 5 min; (ii) 35 cycles of 95 ℃

for 30 sec, 53.5 ℃ for 30 sec, and 72 ℃ for 45 sec; and (iii) 72 ℃ for 5 min. PCR was carried out using Swift maxi thermal cycler (ESCO).

3.2.8.3. Agarose gel electrophoresis

For the visualisation of the PCR products, agarose gel electrophoresis was used. The concentration of agarose gel was 1.5 %. For the preparation of the gel 0.5x TBE buffer was applied. After measuring the required quantity of the agarose powder and the buffer, the solution was heated in a microwave oven. When it became transparent, the gel was cooled to approximately 55-60 °C, 1 μL gel dye was added to every 30 mL of the solution. The gel was poured in a tray during the casting process, and the comb was placed in it. After solidification, the comb was removed, and the samples were pipetted in the wells. The tank (in which the tray was placed before pipetting) was covered with a lid and plugged into the voltage source to run the electrophoresis at 80 V for 90 minutes.

3.2.8.4. Sequence analysis

The amplified DNA segments were purified using the Monarch PCR and DNA Cleanup Kit (New England BioLabs), and sequenced by BIOMI Ltd. (Gödöllő, Hungary). For sequencing, the NL1 primer was used. The sequence data were analysed by Mega11 software, and for identifying the standard nucleotide BLAST (National Center for Biotechnology Information) was used.

4. RESULTS AND DISCUSSION

This Graduate Thesis research was conducted in order to gain insight into the interactions between yeasts and probiotic bacteria. First of all, 16 yeast strains (Table 3.; Chapter 4.1.) and five probiotic bacteria (Figure 1.; Chapter 4.1.) were isolated. Fifteen different media were prepared and inoculated with all yeast isolates in order to determine the optimal growth medium for each of them (Table 5.; Chapter 4.2.). Bacteria isolated from the probiotic preparation were identified by MALDI-TOF (Table 6.; Chapter 4.3.), while the cell morphology of the yeasts was observed under the microscope (Table 7.; Chapter 4.4.).

It was necessary to find a proper growth medium suitable for both yeasts and bacteria to inspect interactions among them (Table 9.; Chapter 4.5.). The next step was the contact inhibition assay. This test aimed to discover which yeast(s) inhibit(s) the growth of bacteria, and vice versa. The results of this experiment exhibited an antagonistic relationship between the bacterium *Bacillus clausii* and two yeast isolates (Figure 2.; Chapter 4.6.).

In many times cell-free cultivation media contain active substances that can inhibit the growth of other microorganisms. Therefore, cell-free cultivation media of all isolates were prepared and tested their potential antagonistic effect (Chapter 4.8.). However, in this case, no inhibition zones were observed.

Regarding results obtained in contact inhibition assay (Figure 2.; Chapter 4.6.) further aim was to identify the yeast isolates. Thus, PCR amplified 28S rDNA segments were sequenced (results shown in Chapter 4.7.). Finally, isolates were co-cultured to confirm antagonistic relations between *Bacillus clausii* and the identified yeasts, *Debarymoyces hansenii* (Table 10.; Chapter 4.9).

4.1. SAMPLES SPECIFICATION

Although three samples were used for the isolation of the yeasts, all isolates (16 of them) were obtained from pear (isolation from yoghurt and soft cheese was not successful). As it is described in chapter 3.2.1., some of them were isolated on DRBC plates, while others on BSA plates. They were named regarding the applied medium for isolation: those which were isolated from DRBC plates are coded with "D", and those which were isolated from BSA plates are indicated by "B". All isolates were transferred to WL agar plates in order to categorise them

depending on their colony morphological characteristics. They were grouped e.g. based on their colony colour: red, pink, light pink, blue and green (Table 3.).

Table 3. The sixteen yeast isolates and their categorisation based on colony morphological characteristics

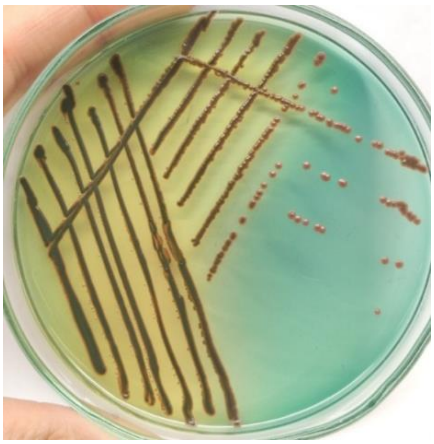
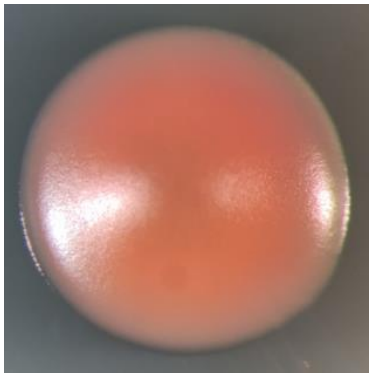
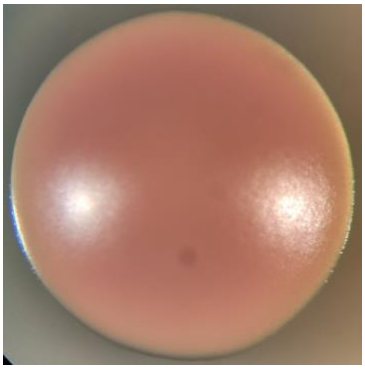
GROUP	CODE	PHOTOS OF PLATES AND COLONIES		DESCRIPTION
RED	D2			Size: tiny colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: red Medium: changed to yellow
	B3			Size: small colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: purple Medium: changed to yellow

Table 3. The sixteen yeast isolates and their categorisation based on colony morphological characteristics (continuation)

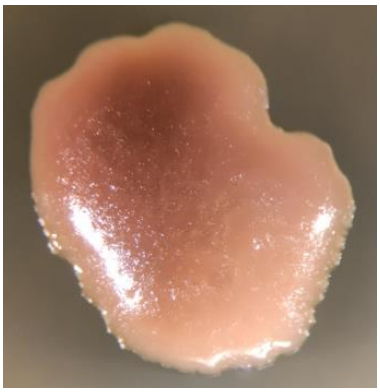
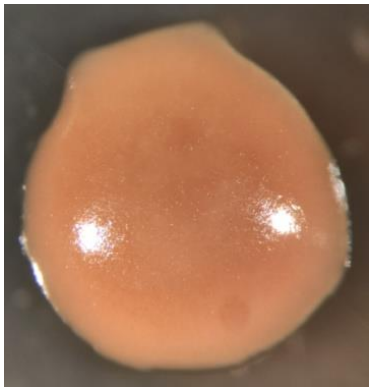
GROUP	CODE	PHOTOS OF PLATES AND COLONIES		DESCRIPTION
PINK	D1			Size: big colonies Form: irregular Elevation: raised Surface: shiny and rough Optical property: opaque Consistency: viscid Pigmentation: dark pink Medium: changed to yellow
	B2			Size: big colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: viscid Pigmentation: peachy pink Medium: changed to yellow
	B4			Size: big colonies Form: circular Elevation: raised and pointed in the middle Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: pink Medium: changed to yellow

Table 3. The sixteen yeast isolates and their categorisation based on colony morphological characteristics (continuation)

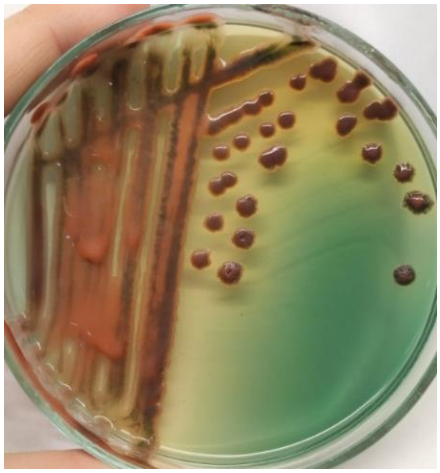
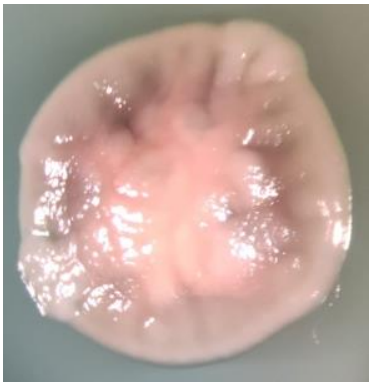
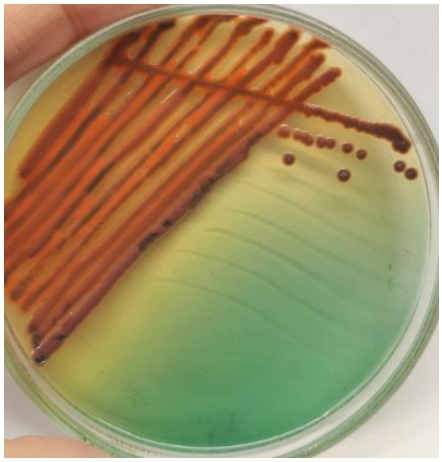
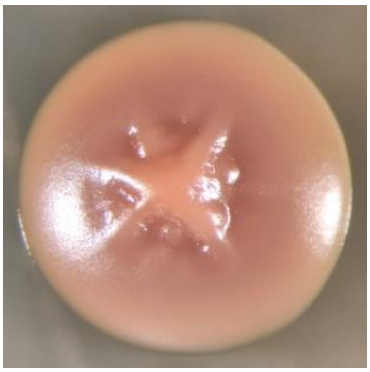
GROUP	CODE	PHOTOS OF PLATES AND COLONIES		DESCRIPTION
PINK	B9			Size: big colonies Form: circular/irregular Elevation: raised Surface: shiny and rough Optical property: opaque Consistency: viscid Pigmentation: dark pink Medium: changed to yellow
	B12			Size: small colonies Form: circular Elevation: raised Surface: shiny and smooth, a bit rough on the top Optical property: opaque Consistency: buttery Pigmentation: pink Medium: changed to yellow
LIGHT PINK	D3			Size: small colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: light pink to wheat Medium: changed to yellow

Table 3. The sixteen yeast isolates and their categorisation based on colony morphological characteristics (continuation)

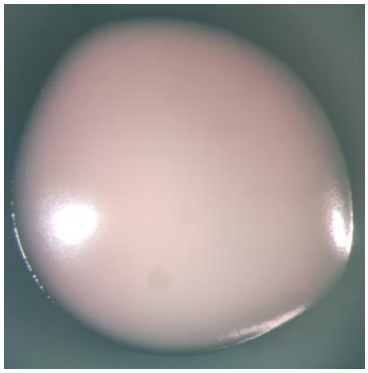
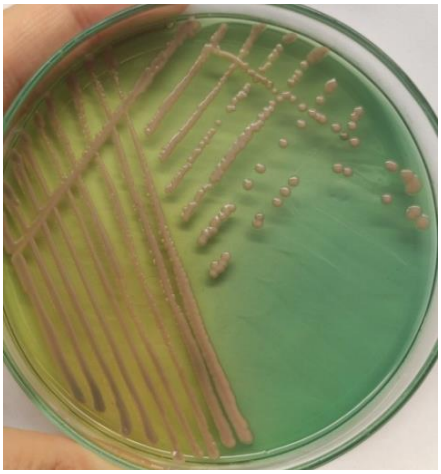
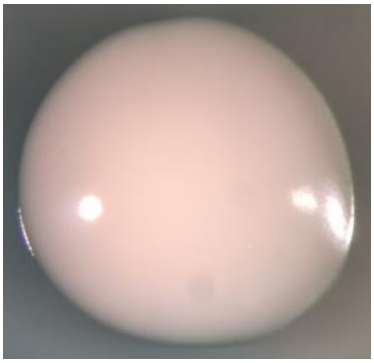
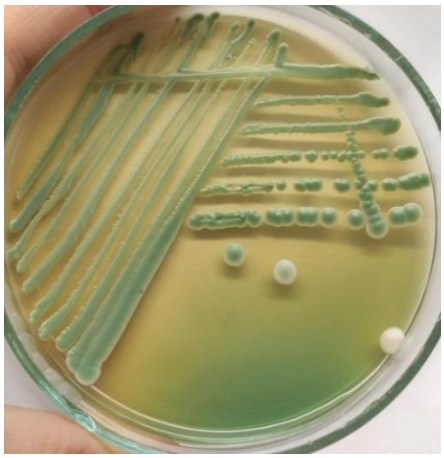
GROUP	CODE	PHOTOS OF PLATES AND COLONIES		DESCRIPTION
LIGHT PINK	B7			Size: small colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: baby pink Medium: changed mildy to yellow
	B11			Size: small colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: wheat Medium: changed to yellow
GREEN	D4			Size: big colonies Form: circular Elevation: raised Surface: dull and smooth Optical property: opaque Consistency: buttery Pigmentation: green Medium: changed to yellow

Table 3. The sixteen yeast isolates and their categorisation based on colony morphological characteristics (continuation)

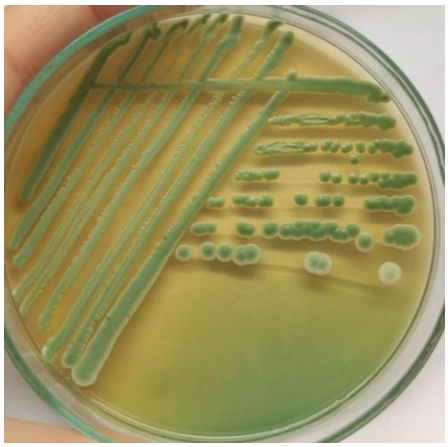
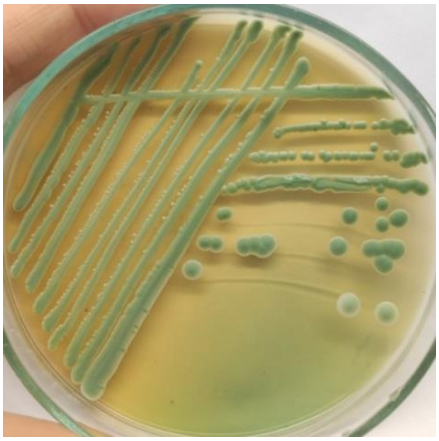
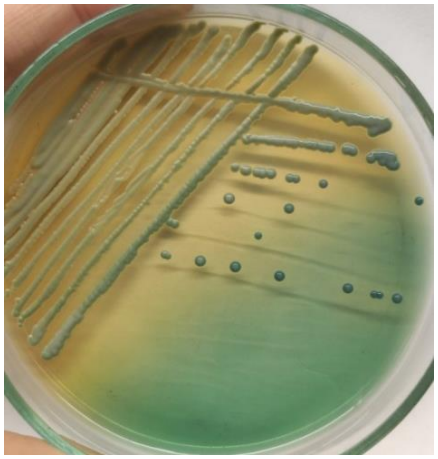
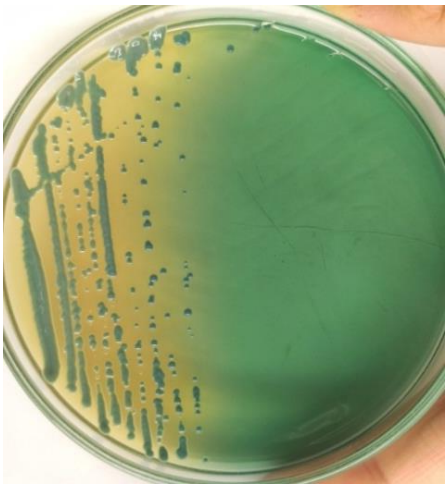
GROUP	CODE	PHOTOS OF PLATES AND COLONIES		DESCRIPTION
GREEN	B5			Size: big colonies Form: circular Elevation: raised Surface: dull and smooth Optical property: opaque Consistency: buttery Pigmentation: green Medium: changed to yellow
	B8			Size: big colonies Form: circular Elevation: raised Surface: rough Optical property: opaque Consistency: dry Pigmentation: green Medium: changed to yellow
	B10			Size: big colonies Form: circular Elevation: raised Surface: dull and smooth Optical property: opaque Consistency: buttery Pigmentation: green Medium: changed to yellow

Table 3. The sixteen yeast isolates and their categorisation based on colony morphological characteristics (continuation)

GROUP	CODE	PHOTOS OF PLATES AND COLONIES		DESCRIPTION
BLUE	D5			Size: small colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: blue Medium: changed to yellow
	B13			Size: tiny colonies Form: circular Elevation: raised Surface: shiny and smooth Optical property: opaque Consistency: buttery Pigmentation: dark blue Medium: changed to yellow

Modified MRS (mMRS) agar was used for the differentiation of each LAB from a mixed culture. Due to bromophenol blue, it was possible to distinguish and isolate four different colonies. They were marked with "A", "B", "C", and "D" (Figure 1.) and described in Table 4. This medium was explicitly developed for lactic acid-producing bacteria. Lee and Lee (2008) designed this formula to make the isolation and enumeration of each LAB from mixed cultures easier. The huge advantage is that this medium supports the growth of all LAB, and even *Bifidobacterium* sp. In addition, mMRS is a better alternative than some recommended media (e.g. agar with bromocresol purple), because it shortens the incubation time of bacteria and enables colonies to grow bigger (helps in the enumeration).

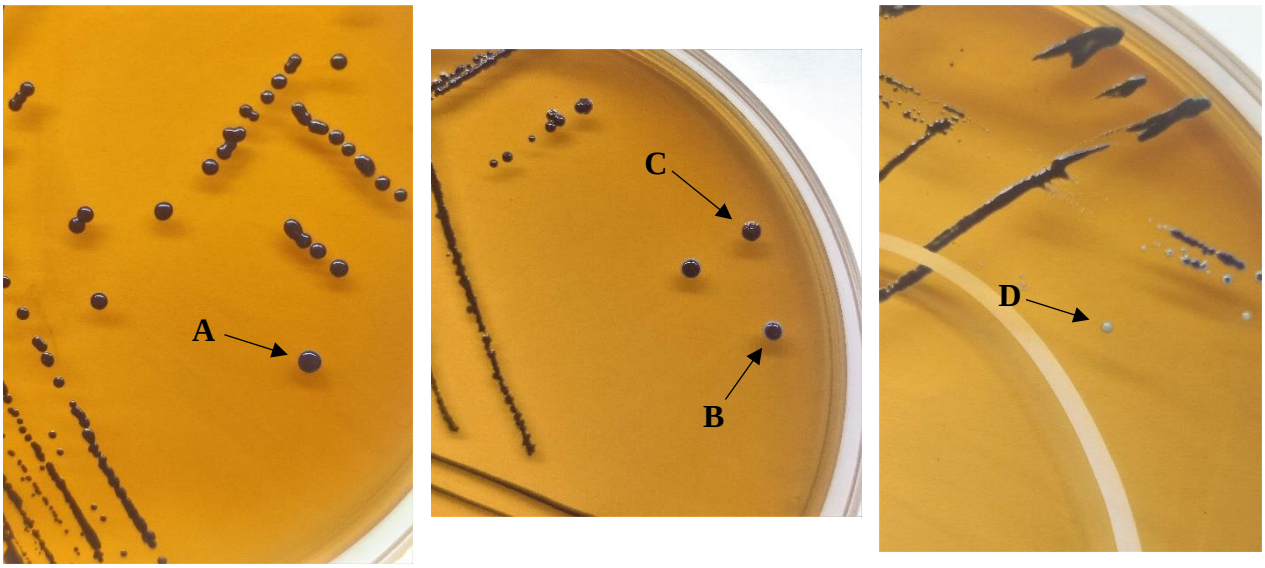


Figure 1. Colony morphology of LAB strains marked A, B, C, and D isolated by cultivation on mMRS agar

Table 4. Description of isolated LAB based on different characteristics of the colonies

CHARACTERISTICS	LAB			
	A	B	C	D
Size	Big colonies	Medium colonies	Medium colonies	Small colonies
Form	Circular	Circular	Circular	Circular
Surface	Shiny	Shiny	Shiny	Shiny
Optical property	Opaque	Opaque	Opaque	Opaque
Consistency	Buttery	Buttery	Buttery	Buttery
Pigmentation	Dark blue	Dark blue	Dark blue	Light blue
Margin	Entire	Entire	Entire	Entire
Elevation	Raised	Raised	Umbonate	Raised

4.2. CULTIVATION OF THE ISOLATED YEASTS ON DIFFERENT MEDIA

Since five different groups of yeast isolates were recognised (Chapter 4.1.), it was assumed that a different medium would suit better for some of them. Thus, 15 media were prepared and inoculated with 16 yeast isolates. Nine isolates did not grow on any media, so they were not shown in Table 5.

Yeast D2 did not grow on the following media: Pear 2x, TSA, TSA + $FeCl_3$, Yeast & Mould Agar, and Standard Nutrient I agar. In addition, Pear 10x and TSA+$FeSO_4$ were not suitable for yeast D3, while all examined media supported the growth of D4, B5, B7, B10 and B11 isolates.

Pear 4x, O.G.Y.E., OGY-agar, Malt Extract Agar, YEPD, PDA and Nutrient Agar supported the growth of all isolates (Table 5.). However, YEPD was chosen as a medium for further experiments since yeast colonies were more numerous and bigger than colonies on other media.

Table 5. Photographs of seven yeast isolates on 15 different media

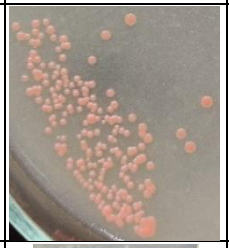
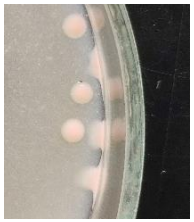
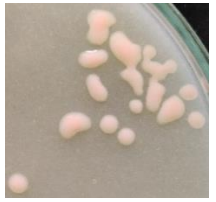
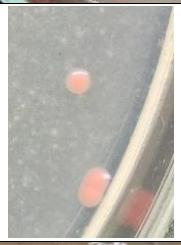
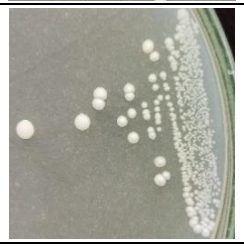
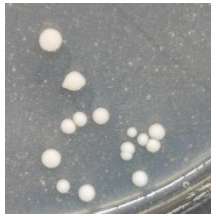
MEDIUM	YEASTS						
	D2	D3	D4	B5	B7	B10	B11
Pear 2x	/						
Pear 4x							
Pear 10x		/					
O.G.Y.E.							

Table 5. Photographs of seven yeast isolates on 15 different media (continuation)

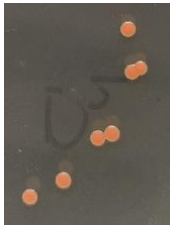
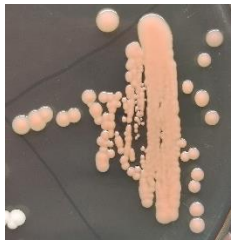
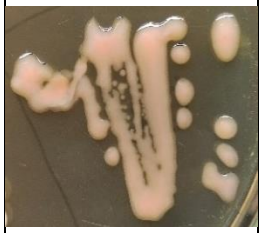
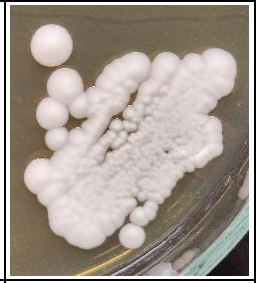
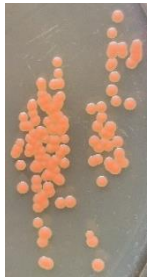
MEDIUM	YEASTS						
	D2	D3	D4	B5	B7	B10	B11
OGY-agar							
Malt Extract Agar							
YEPD							

Table 5. Photographs of seven yeast isolates on 15 different media (continuation)

MEDIUM	YEASTS						
	D2	D3	D4	B5	B7	B10	B11
TSA	/						
TSA+$FeSO_4$		/					
TSA+$FeCl_3$	/						

Table 5. Photographs of seven yeast isolates on 15 different media (continuation)

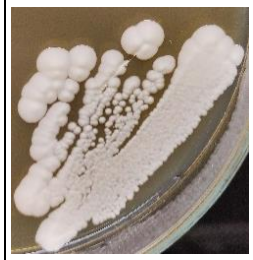
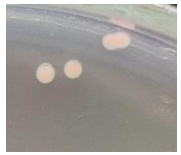
MEDIUM	YEASTS						
	D2	D3	D4	B5	B7	B10	B11
Yeast & Mould Agar	/						
PDA							
Nutrient agar							

Table 5. Photographs of seven yeast isolates on 15 different media (continuation)

MEDIUM	YEASTS						
	D2	D3	D4	B5	B7	B10	B11
Standard Nutrient I agar	/						
BSA	/						

4.3. MALDI-TOF MS ANALYSIS FOR IDENTIFICATION OF THE ISOLATES

All 20 isolates were subjected to analysis by MALDI-TOF. Yeasts could not be effectively identified by MALDI-TOF MS except B10 isolate. The best match for this yeast was *Candida famata* (score value 1.70), while the second match was the same organism with score value 1.68. Although this method of identification is very popular because of its rapidity, especially in clinical samples, limited databases make the identification complicated. Until spectra libraries are improved, and better organised, more accurate yeast identification strategy is DNA sequencing (Richard and Sokol, 2019).

The identification of LAB strains, althought the results were not precise and had multiple matches, was more efficent. Microorganisms were determined by comparing MALDI-TOF MS results and probiotic bacteria listed on the package of Protexin Junior +C (Table 2.).

mMRS isolated strains from the commercial probiotic preparation Protexin Junior +C were taken into account; based on these data, Table 6. was constructed. There were only one or two matches that could be considered. The match with the higest score value is regarded as the identity of bacteria. MALDI-TOF MS results for isolates A, B, and D were the exact (first match – *L. rhamnosus* and second match – *L. paracasei*). However, colonies differed in size and colour (Figure 1.; Table 4.). D colony was the smallest and light blue. The size of B was medium, and the colour was dark blue. However, the edges of this colony were light blue. Finally, A was the biggest and utterly dark blue. Therefore, it could be concluded that these three isolates are *L. rhamnosus*, but in different growth phases.

Table 6. Results of MALDI-TOF MS identification in case of LAB isolated from Protexin Junior + C

ISOLATE	ORGANISM (FIRST MATCH)	SCORE VALUE	ORGANISM (SECOND MATCH)	SCORE VALUE
A	*Lactobacillus rhamnosus*	1.38	*Lactobacillus paracasei*	1.32
B	*Lactobacillus rhamnosus*	1.69	*Lactobacillus paracasei*	1.52
C	*Lactobacillus acidophilus*	1.75	/	/
D	*Lactobacillus rhamnosus*	1.98	*Lactobacillus paracasei*	1.43

4.4. MORPHOLOGY OF THE YEAST CELLS

The cell morphology of all presumptive yeast isolates was evaluated by light microscope. This confirmed that those 16 microorganisms were yeasts, and that can be distinguished by some properties described in Table 7.

Table 7. Morphological properties of the isolated yeast cells

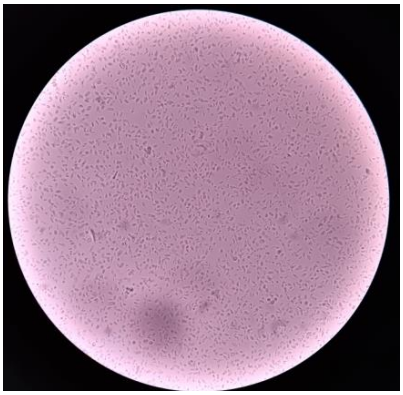
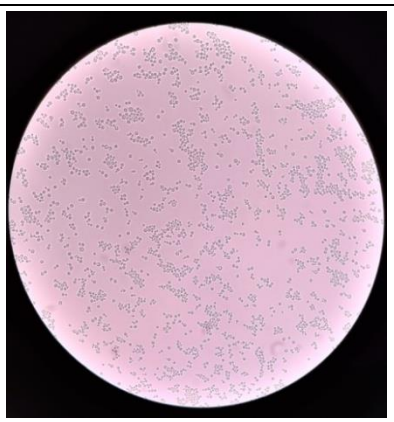
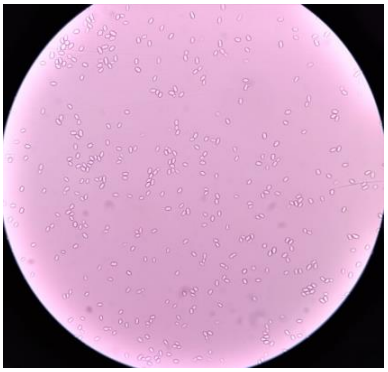
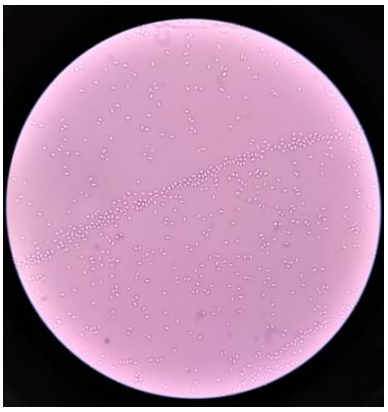
Photo	Description	Photo	Description
D1		B5	
	Cell shape: apiculate Cell division pattern: end-budding Cell clustering patterns: single cells Vacuolation: large vacuoles		Cell shape: circular/ovoid Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: large vacuoles
D2		B7	
	Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles		Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles

Table 7. Morphological properties of the isolated yeast cells (continuation)

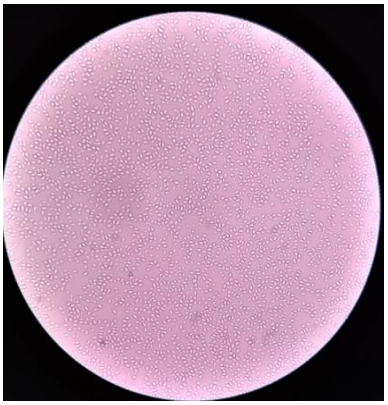
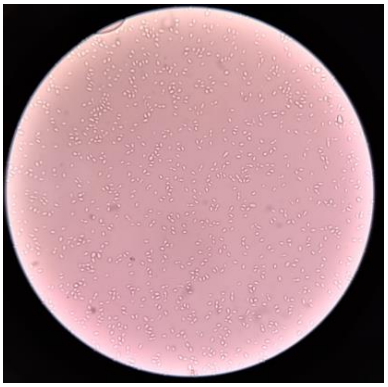
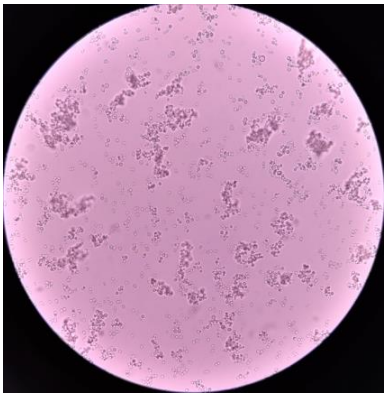
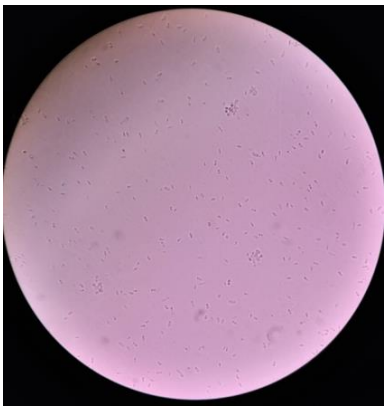
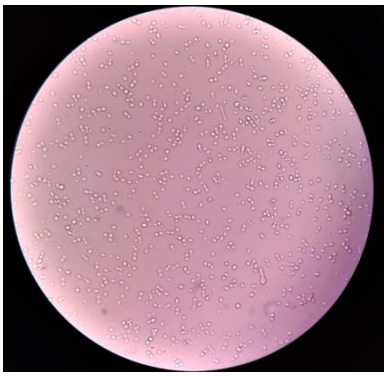
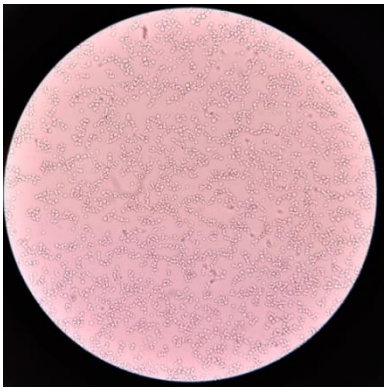
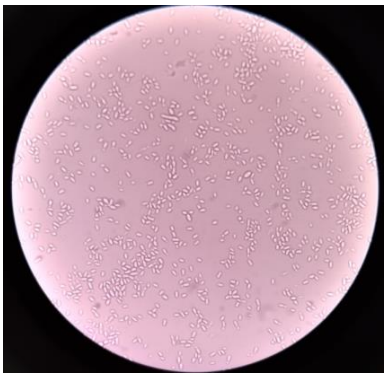
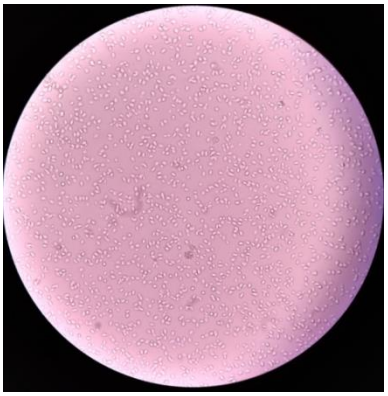
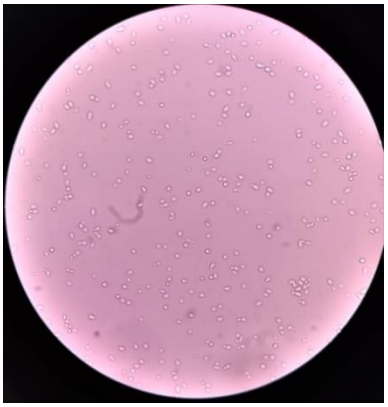
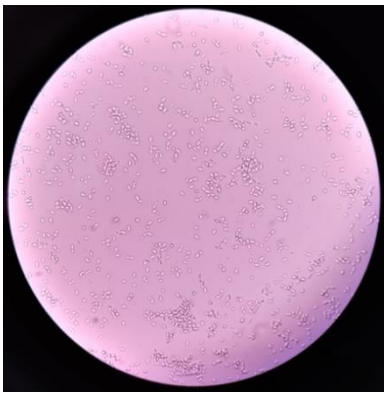
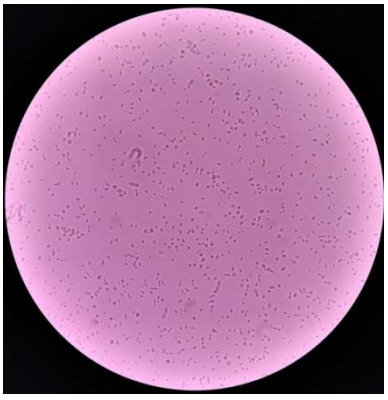
Photo	Description	Photo	Description
D3		B8	
	Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles		Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles
D4		B9	
	Cell shape: circular/ovoid Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles		Cell shape: elongated Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: multiple, small vacuoles
D5		B10	
	Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles		Cell shape: circular/ovoid Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: small vacuoles

Table 7. Morphological properties of the isolated yeast cells (continuation)

Photo	Description	Photo	Description
B2		B11	
	Cell shape: elongated Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: no visible vacuoles		Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: multiple, small vacuoles
B3		B12	
	Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: small vacuoles		Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: multiple, small vacuoles
B4		B13	
	Cell shape: elongated Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: large vacuoles		Cell shape: apiculate Cell division pattern: budding yeast Cell clustering patterns: single cells Vacuolation: multiple, small vacuoles

Four parameters describe cell morphology: cell shape, cell division pattern, cell clustering pattern and vacuolation. The cell clustering pattern was the same for all isolates, while the cell division pattern only differed for yeast D1, which divides by end-budding. Based on cell shape and vacuolation, three groups could be distinguished. This time they were grouped depending on cell shape (Table 8.).

Table 8. The categorisation of 16 yeast isolates based on their cell shape

CELL SHAPE		
apiculate	circular/oval	elongated
D1, D2, D3, D5, B3, B7, B8, B11, B12, B13	D4, B5, B10	B2, B4, B9

4.5. DETERMINATION OF MEDIUM AND CONDITIONS SUITABLE FOR CULTURING OF YEASTS AND PROBIOTIC BACTERIA

This experiment was conducted as it is described in chapter 3.2.5.1. Formed colonies were counted, and the results were summarized in Table 9. MRS agar proved to be suitable for interaction studies in case of yeasts and LAB, while TSA was found as a good candidate for analysing the interactions of yeasts and *B. clausii*. Although more colonies of *B. clausii* were counted when incubation was carried out under semi-anaerobic conditions, the colonies were smaller than those incubated at aerobic conditions, so it was concluded that lack of oxygen suppresses its growth. According to Marseglia et al. (2007) *B. clausii* is an aerobic bacterium, and my observation is in correspondence with this. As it can be seen in Table 9., more LAB colonies were counted on TSA, but they were bigger on MRS agar plates, which was the indicator of better growth support. The most widely used media for isolation and enumeration of LAB are Ragosa agar, MRS agar, Lactobacilli selective agar, TSA and plate count agar with bromocresol purple. However, MRS agar is a standard and simple option for isolating and cultivating LAB (Lee and Lee, 2008).

Table 9. Numbers of colonies grown on different media under diverse culturing conditions

MICROORGANISM	MEDIUM	30 °C, SEMI-ANAEROBIC	30 °C, AEROBIC
Yeast B10	MRS	239	230
	YEPD	268	310
	TSA	400	361
LAB D	MRS	21	42
	YEPD	1	2
	TSA	62	48
Bacillus clausii	MRS	0	0
	YEPD	0	0
	TSA	12	4

4.6. GROWTH INHIBITION STUDIES

4.6.1. Inhibition of probiotic bacteria by the yeasts

Here are described results of the experiment reported in chapter 3.2.5.2. In this experiment, certain difficulties were encountered. Namely, only LAB C (*Lactobacillus acidophilus*) was growing well, and LAB A, B and D did not cover the plate (lawn was not formed). Moreover, only six yeasts (D3, D4, B5, B7, B10 and B11) could grow during the incubation with bacteria. Therefore, only plates overlaid with LAB C and *B. clausii* could have been taken into account.

In the case of *Lactobacillus acidophilus*, there were no inhibition zones, and it can be considered that none of the studied yeasts were able to inhibit this bacterium.

However, weak inhibition zones were visible around yeasts B5 and B10 inoculated on TSA plate overlaid with *B. clausii* (Figure 2.), implying how these yeasts inhibit the growth of bacterium *B. clausii*. Until now, most surveys were conducted in order to investigate inhibition of yeasts by bacteria, so this result can be considered as a step forward in better understanding of vice versa interactions.

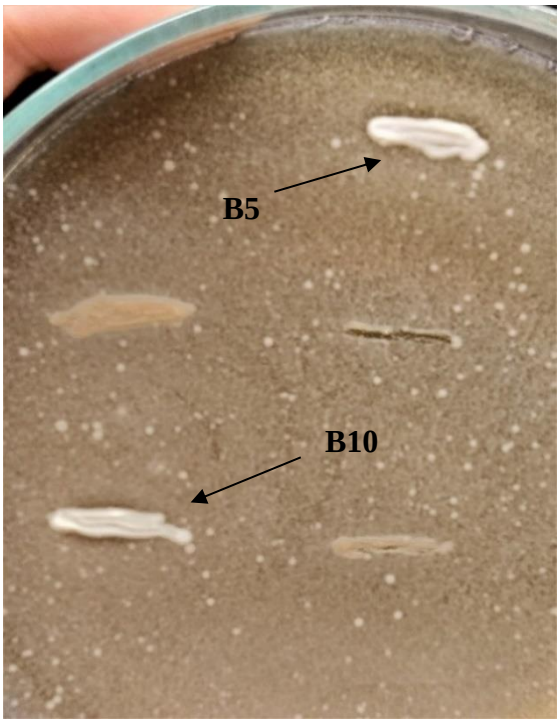


Figure 2. Inhibition zones around yeasts B5 and B10 on TSA plate massively inoculated by *B. clausii*

4.6.2. Inhibition of yeasts by probiotic bacteria

The results of this experiment (chapter 3.2.5.3.) were also affected by the problems described in the latest chapter. This time, only yeasts D4, B5 and B10 have covered TSA plates completely. After the joint incubation of yeast and bacteria, none of the LAB grew. Thus, it was only possible to evaluate the interaction between the three yeasts and *B. clausii*. This time, no inhibition zones could be seen. According to this, *B. clausii* could not inhibit yeasts D4, B5 and B10.

4.7. IDENTIFICATION OF THE B5 AND B10 YEAST ISOLATES BY DNA SEQUENCING

After the results obtained with the contact inhibition test, where it was found that yeasts B5 and B10 inhibited the growth of *B. clausii*, it was essential to identify those fungi. Since MALDI-TOF MS analysis was unsuccessful (except for B10, which was identified as *Candida famata*), amplified DNA samples were sent for sequencing (described in detail in chapter 3.2.8.).

Result of molecular identification in the case of B5 yeast strain:

>ANA-2_6#NL1

GAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGAAGAAGGTAACTTTGGAGTTGGCTCTTGTCT
ATGTTCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATTCTATGT
AAAGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAA
AGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTG
AAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGACTTGGTATTT
TGCGATCCTTTCCTTCTTGGTTGGGTTCCTCGCAGCTTACTGGGCCAGCATCGGTTTGGATGGTAGG
ATAATGACTAAGGAATGTGGCTCTACTTCGGTGGAGTGTTATAGCCTTGGTTGATACTGCCTGTCTA
GACCGAGGACTGCGTCTTTTGACTAGGATGTTGGCATAATGATCTTAAGCCACCCGTCTTG

Identified species: ***Debaryomyces hansenii*** **(100 %)**

Result of molecular identification in the case of B10 yeast strain:

>ANA-3_7#NL1

CTTTTCCTCCGCTTATTGATATGCATCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTGCG
ATGAGATGCCCAATTCTATGTAAAGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAG
TGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGAT
GGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGG
CTTGAGATCAGACTTGGTATTTTGCGATCCTTTCCTTCTTGGTTGGGTTCCTCGCAGCTTACTGGGCC
AGCATCGGTTTGGATGGTAGGATAATGACTAAGGAATGTGGCTCTACTTCGGTGGAGTGTTATAGC
CTTGGTTGATACTGCCTGTCTAGACCGAGGACTGCGTCTTTTGACTAGGATGTTGGCATAATGATCT
TAAGCCACCCGTCTTG

Identified species: ***Debaryomyces hansenii*** **(100 %)**

Both isolates were identified as the same microorganism, *Debaryomyces hansenii*. However, MALDI-TOF MS result for B10 was *Candida famata*. Following the study of Dmytruk et al. (2006) *Candida famata* is an anamorph of the yeast *Debaryomyces hansenii*. Thus, it can be considered that MALDI-TOF MS results correspond with those obtained by DNA sequencing.

4.8. INHIBITORY EFFECT OF CELL-FREE CULTIVATION MEDIA

After incubation time, no clearing zones were visible on any inoculated plates, which shows that extracellular metabolites of yeasts D4, B5 and B10 did not inhibit the growth of *L. acidophilus* and *B. clausii.* Furthermore, the cell-free cultivation media of bacteria did not inhibit the growth of yeasts D4, B5 and B10, as well. Vilela et al. (2015) demonstrated that cultivation media of *L. acidophilus* inhibited biofilm formation of *C. albicans.* Similar results were shown in the report of Matsubara et al. (2016), but they used *Lactobacillus rhamnosus* for

inhibition. Another group of scientists (Cizeikiene et al., 2013) used several LAB cultivation media for well diffusion assay to investigate the antagonistic effect on many pathogenic bacteria, fungi and yeasts. *Lactobacillus sakei* and *Pediococcus pentosaceus* were found to have fungistatic activities against *Debaryomyces hansenii*. In addition, these LAB cultivation media inhibited *Candida parapsilosis* along with mould *Fusarium culmorum*. Besides *D. hansenii,* LAB growth media acted as fungistatic against moulds *Penicillium expansum*, *Penicillium chrysogenum, Aspergillus niger, Aspergillus fumigatus*, and *Aspergillus versicolor*.

4.9. CO-CULTURING OF *BACILLUS CLAUSII* AND *DEBARYOMYCES HANSENII* B5 AND B10

Results of the contact inhibition test demonstrated weak inhibition zones of bacterial growth around yeast isolates B5 and B10. To confirm antagonistic relationship between these yeasts and *B. clausii* co-culturing experiment was conducted (Chapter 3.2.7.). After incubation time colonies were counted and calculated (Table 10.).

Table 10. Results of co-culturing study in case of *B. clausii* and yeast strains B5 and B10.

SAMPLES	Concentration in the original suspension (CFU/mL)	
	Time 0	After 48 h
B. clausii (on TSA)	$9.8x10^4$	$6x10^6$
B5 (on TSA)	$8x10^3$	$3.1x10^6$
B10 (on TSA)	$1.1x10^4$	$2.1x10^6$
B. clausii + B5 (on TSA+nystatin)	$7.2x10^4$	$4.9x10^4$
B. clausii + **B5** (on RBC)	$6x10^3$	$9.08x10^5$
B. clausii+ B10 (on TSA+nystatin)	$8.3x10^4$	$1.43x10^7$
B. clausii + **B10** (on RBC)	$1.8x10^4$	$5.88x10^5$

Nystatin was added to TSA in order to prevent the growth of fungi. Thus, all colonies counted on such medium were bacterial. The concentration of yeast cells was determined by inoculation on RBC plates. Chloramphenicol blocked the growth of bacteria allowing only yeast to form colonies.

During the 48 hours of incubation time approximately two orders of magnitude increase was detected both for *B. clausii* and the yeast strains.

Co-culture of *B. clausii* and B5:

The same suspension was inoculated on TSA+nystatin plate and RBC agar plate. *B. clausii* colonies were counted on a nystatin-containing TSA plate. Before incubation, the concentration of bacterial cells in the mixture was $7.2x10^4$ CFU/mL, and after incubation, $4.9x10^4$ CFU/mL. The concentration change of the yeast cells in the mixture was determined on RBC agar plate ($6x10^3$ CFU/mL before incubation and $9.08x10^5$ CFU/mL after incubation). The bacterium concentration did not change significantly, or even a small decrease could be observed. On the other hand, the concentration of yeast cells increased by two orders of magnitude. Since the concentration of bacteria in the mixture did not increase after the 48-h incubation, it can be concluded that B5 isolate inhibited the growth of *B. clausii*.

Co-culture of *B. clausii* and B10:

Bacterial colonies were counted on TSA+nystatin medium, while the yeast colonies were counted on RBC agar plate. At the beginning of the experiment concentration of microorganisms in the mixture was $8.3x10^4$ CFU/mL (*B. clausii*) and $1.8x10^4$ CFU/mL (B10). After the 48-h incubation time, their concentration changed to $1.43x10^7$ CFU/mL and $5.88x10^5$ CFU/mL, respectively. This co-culturing resulted differently than the previous one. Namely, the cell concentration of both organisms in the mixture increased. Thus, no inhibition of *B. clausii* by isolate B10 occurred.

Although both yeasts were identified as *Debaryomyces hansenii* (Chapter 4.7.), this experiment showed how only B5 inhibited *B. clausii*. These results highlight the difference at strain level between them, and it can be concluded, that *D. hansenii* B5 is an antagonistic strain of *B. clausii* probiotic bacterium.

Previous studies reported that *D. hansenii* inhibits many pathogens (Chapter 2.5.1.). Research done in 1989 by Meisel *et al.* investigated several starters against the pathogen *S. aureus* in dry sausages. One of the microorganisms able to suppress the growth of this bacterium was *D. hansenii*. Moreover, when it was combined with the other meat starters, inhibition was remarkably effective. Furthermore, killer toxins secreted by *D. hansenii* efficiently inhibited pathogenic bacteria *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus pyogenes* and yeasts *C. albicans* and *Candida neoformans*. The same toxin inhibited the growth of fungal mycelia of two human pathogens (*Trichophyton rubrum* and *Trichophyton concentricum*) and two plant pathogens (*Alternaria alternata* and *Curvularia lunata*) (Al-Qaysi et al.). Iacumin et al. (2020) also reported the benefit of *D. hansenii* in preventing mould

spoilage of dry-cured ham. Namely, *Aspergillus westerdijkiae* produces ochratoxin A, which represents a health risk for consumers. Antagonistic activities of *D. hansenii* on the growth of this mould were proven *in vitro* and *in situ* and were enhanced with the addition of *Lactobacillus buchneri*.

Even though many previous studies describe antimicrobial activity against diverse microorganisms, literature concerning to relationship between *D. hansenii* and *B. clausii* could not be found. Thus, this is the first time inhibition of *B. clausii* by *D. hansenii* has been reported.

5. CONCLUSIONS

Based on the results presented in this Graduate Thesis, the following conclusions can be drawn:

1. MALDI-TOF MS identification of yeast species is limited since spectra libraries do not offer a wide spectrum of data on this matter. Thus, the more accurate method for yeast identification is DNA sequencing.

2. Tested yeast cultures grew on almost all prepared media, but the optimal results in terms of growth were obtained during the cultivation on YEPD agar. Thus, it can be concluded that YEPD agar can be an optimal and universal choice for the isolation and maintenance of yeasts.

3. It was established how yeast *Debaryomyces hansenii* inhibits the growth of the probiotic bacterium *Bacillus clausii*. Antagonism was recognised in the contact-inhibition assay and later confirmed by co-culturing test. This unique finding greatly impacts and enhances the knowledge of the interactions between yeasts and probiotic bacteria.

6. REFERENCES

Al-Qaysi SAS, Al-Haideri H, Thabit ZA, Al-Kubaisy WHAA-R, Ibrahim JAA-R (2017) Production, Characterization, and Antimicrobial Activity of Mycocin Produced by *Debaryomyces hansenii* DSMZ70238. *Int J Microbiol* **2017**, 1–9. https://doi.org/10.1155/2017/2605382

Anjum N, Maqsood S, Masud T, Ahmad A, Sohail A, Momin A (2014) *Lactobacillus acidophilus*: Characterization of the Species and Application in Food Production. *Crit Rev Food Sci Nutr* **54**(9), 1241-1251. https://doi.org/10.1080/10408398.2011.62116

Breuer U, Harms H (2006) *Debaryomyces hansenii* — an extremophilic yeast with biotechnological potential. *Yeast* **23**(6), 415-437. https://doi.org/10.1002/yea.1374

Cizeikiene D, Juodeikiene G, Paskevicius A, Bartkiene E (2013) Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control* **31**(2), 539–545. https://doi.org/10.1016/j.foodcont.2012.12.004

Cucgini C, Calfee MW, Farrow JM, Morales DK, Pesci EC, Hogan DA (2007) Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. *Mol Microbiol* **65**(4), 896-906. https://doi.org/10.1111/j.1365-2958.2007.05840.x

Cui L, Morris A, Ghedin E (2013) The human mycobiome in health and disease. *Genome Med* **5**, 63. https://doi.org/10.1186/gm467

Dmytruk KV, Voronovsky AY, Sibirny AA (2006) Insertion mutagenesis of the yeast *Candida famata* (*Debaryomyces hansenii*) by random integration of linear DNA fragments. *Curr Genet* **50**(3), 183-191. https://doi.org/10.1007/s00294-006-0083-0

Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C, Panwar H (2017) *Bacillus* As Potential Probiotics: Status, Concerns, and Future Perspectives. *Front Microbiol* https://doi:10.3389/fmicb.2017.01490

Fakruddin M, Hossain MN, Ahmed MM (2017) Antimicrobial and antioxidant activities of *Saccharomyces cerevisiae* IFST062013, a potential probiotic. *BMC Complement Altern Med* **17**, 64. https://doi.org/10.1186/s12906-017-1591-9

Faruque SM, Albert MJ, Mekalanos JJ (1998) Epidemiology, Genetics, and Ecology of Toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* **62**(4), 1301-1314. https://doi.org/10.1128/mmbr.62.4.1301-1314.1998

Fontana L, Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gil A (2013) Sources, isolation, characterisation and evaluation of probiotics. *Br J Nutr* **109**(S2), S35–S50. https://doi.org/10.1017/s0007114512004011

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. (2014) The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* **11**, 506–514. https://doi.org/10.1038/nrgastro.2014.66

Hoarau G, Mukherjee PK, Gower-Rousseau C, Hager C, Chandra J, Retuerto MA, et al. (2016) Bacteriome and Mycobiome Interactions Underscore Microbial Dysbiosis in Familial Crohn's Disease. *MBio* **7**(5), e01250-16. https://doi.org/10.1128/mBio.01250-16

Homayouni-Rad A, Azizi A, Oroojzadeh P, Pourjafar H (2020) *Kluyveromyces marxianus* as a probiotic yeast: A mini-review. *Curr Nutr Food Sci* **16**(8), 1163-1169. https://doi.org/10.2174/1573401316666200217113230

Huseyin CE, O'Toole PW, Cotter PD, Scanlan PD (2017) Forgotten fungi - the gut mycobiome in human health and disease. *FEMS Microbiol Rev* **41**(4), 479-511. https://doi.org/10.1093/femsre/fuw047

Iacumin L, Arnoldi M, Comi G (2020) Effect of a *Debaryomyces hansenii* and *Lactobacillus buchneri* starter culture on *Aspergillus westerdijkiae* ochratoxin A production and growth during the manufacture of short seasoned dry-cured ham. *Microorganisms* **8**(10), 1623. https://doi.org/10.3390/microorganisms8101623

Janssens Y, Nielandt J, Bronselaer A, Debunne N, Verbeke F, Wynendaele E, et al. (2018) Disbiome database: linking the microbiome to disease. *BMC Microbiol* **18**(1), 1-6. https://doi.org/10.1186/s12866-018-1197-5

Kaźmierczak-Siedlecka K, Ruszkowski J, Fic M, Folwarski M, Makarewicz W (2020) *Saccharomyces boulardii* CNCM I-745: A Non-bacterial Microorganism Used as Probiotic Agent in Supporting Treatment of Selected Diseases. *Curr Microbiol* **77**(9), 1987-1996. https://doi.org/10.1007/s00284-020-02053-9

Kim SK, Guevarra RB, Kim YT, Kwon J, Kim H, Cho JH, et al. (2019) Role of Probiotics in Human Gut Microbiome-Associated Diseases. *J Microbiol Biotechnol* **29**, 1335-1340. https://doi.org/10.4014/jmb.1906.06064

Kim YJ, Yu HH, Park YJ, Lee NK, Paik HD (2020) Anti-biofilm activity of cell-free supernatant of *Saccharomyces cerevisiae* against *Staphylococcus aureus*. *J Microbiol Biotechnol* **30**(12), 1854-1861. https://doi.org/10.4014/jmb.2008.08053

Köhler GA, Assefa S, Reid G (2012) Probiotic interference of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 with the opportunistic fungal pathogen *Candida albicans*. *Infect Dis Obstet Gynecol* **2012**, 636474. https://doi.org/10.1155/2012/636474

Krüger W, Vielreicher S, Kapitan M, Jacobsen ID, Niemiec MJ (2019) Fungal-Bacterial Interactions in Health and Disease. *Pathogens* **8**(2), 70. https://doi.org/10.3390/pathogens8020070

Kumamoto CA, Gresnigt MS, Hube B (2020) The gut, the bad and the harmless: *Candida albicans* as a commensal and opportunistic pathogen in the intestine. *Curr Opin Microbiol* **56**, 7-15. https://doi.org/10.1016/j.mib.2020.05.006

Lane MM, Morrissey JP (2010) *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biol Rev* **24**(1-2), 17-26. https://doi.org/10.1016/j.fbr.2010.01.001

Lara-Hidalgo CE, Hernández-Sánchez H, Hernández-Rodríguez C, Dorantes-Álvarez L (2017) Yeasts in Fermented Foods and their Probiotic Potential. *Austin J Nutr Metab* **4**(1), 1045. https://www.researchgate.net/publication/319528391

LaSarre B, Federle MJ (2013) Exploiting Quorum Sensing To Confuse Bacterial Pathogens. *Microbiol Mol Biol Rev* **77**(1), 73-111. https://doi.org/10.1128/mmbr.00046-12

Lee HM, Lee Y (2008) A differential medium for lactic acid-producing bacteria in a mixed culture. *Lett Appl Microbiol* **46**(6), 676–681. https://doi.org/10.1111/j.1472-765x.2008.02371.x

Lee N-K, Kim W-S, Paik H-D (2019) *Bacillus* strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. *Food Sci Biotechnol* https://doi.org/10.1007/s10068-019-00691-9

Leonhardt I, Spielberg S, Weber M, Albrecht-Eckardt D, Bläss M, Claus R, et al. (2015) The fungal quorum-sensing molecule farnesol activates innate immune cells but suppresses cellular adaptive immunity. *MBio* **6**(2), e00143-15. https://doi.org/10.1128/mBio.00143-15

Lister JL, Horswill AR (2014) *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. *Front Cell Infect Microbiol* **4**, 178. https://doi.org/10.3389/fcimb.2014.00178

Lopetuso LR, Scaldaferri F, Franceschi F, Gasbarrini A (2016). *Bacillus clausii* and gut homeostasis: state of the art and future perspectives. *Expert Rev Gastroenterol Hepatol* **10**(8), 943-948. https://doi.org/10.1080/17474124.2016.1200465

Malka O, Kalson D, Yaniv K, Shafir R, Rajendran M, Ben-David O., et al. (2021). Cross-kingdom inhibition of bacterial virulence and communication by probiotic yeast metabolites. *Microbiome* **9**(1), 1-15. https://doi.org/10.1186/s40168-021-01027-8

Marseglia GL, Tosca M, Cirillo I, Licari A, Leone M, Marseglia A, et al. (2007) Efficacy of *Bacillus clausii* spores in the prevention of recurrent respiratory infections in children: a pilot study. *Ther Clin Risk Manag* **3**(1), 13-17. https://doi.org/10.2147/tcrm.2007.3.1.13

Matsubara VH, Wang Y, Bandara HMHN, Mayer MPA, Samaranayake LP (2016) Probiotic *Lactobacilli* inhibit early stages of *Candida albicans* biofilm development by reducing their growth, cell adhesion, and filamentation. *Appl Microbiol Biotechnol* **100**, 6415-6426. https://doi.org/10.1007/s00253-016-7527-3

Meisel C, Gehlen KH, Fischer A, Hammes WP (1989) Inhibition of the growth of *Staphylococcus aureus* in dry sausages by *Lactobacillus curvatus*, *Micrococcus varians* and *Debaryomyces hansenii*. Food Biotechnol **3**(2), 145–168. https://doi.org/10.1080/08905438909549705

Noverr MC, Huffnagle GB (2004) Regulation of *Candida albicans* Morphogenesis by Fatty Acid Metabolites. *Infect Immun* **72**(11), 6206-6210. https://doi.org/10.1128/IAI.72.11.6206-6210.2004

Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ (2018) Invasive candidiasis. *Nat Rev Dis Primers* **4**(1), 1-20. https://doi.org/10.1038/nrdp.2018.26

Pérez JC (2021) Fungi of the human gut microbiota: Roles and significance. *Int J Med Microbiol* **311**(3), 151490. https://doi.org/10.1016/j.ijmm.2021.151490

Ponomarova O, Gabrielli N, Sévin DC, Mülleder M, Zirngibl K, Bulyha K, et al. (2017) Yeast creates a niche for symbiotic lactic acid bacteria through nitrogen overflow. *Cell Syst* **5**(4), 345-357. https://doi.org/10.1016/j.cels.2017.09.002

Richard ML, Sokol H (2019) The gut mycobiota: insights into analysis, environmental interactions and role in gastrointestinal diseases. *Nat Rev Gastroenterol Hepatol* **16**(6), 331-345. https://doi.org/10.1038/s41575-019-0121-2

Saidi N, Owlia P, Marashi SMA, Saderi H (2019) Inhibitory effect of probiotic yeast *Saccharomyces cerevisiae* on biofilm formation and expression of α-hemolysin and enterotoxin A genes of *Staphylococcus aureus*. *Iran J Microbiol* **11**(3), 246-254. https://doi.org/https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6711867/

Sakarya S, Gunay N (2014) *Saccharomyces boulardii* expresses neuraminidase activity selective for α2, 3-linked sialic acid that decreases *Helicobacter pylori* adhesion to host cells. *APMIS* **122**(10), 941-950. https://doi.org/10.1111/apm.12237

Shruthi B, Deepa N, Somashekaraiah R, Adithi G, Divyashree S, Sreenivasa MY (2022) Exploring biotechnological and functional characteristics of probiotic yeasts: A review. *Biotechnol Rep* **34**, e00716. https://doi.org/10.1016/j.btre.2022.e00716

Sudbery PE (2011) Growth of *Candida albicans* hyphae. *Nat Rev Microbiol* **9**(10), 737–748. https://doi.org/10.1038/nrmicro2636

Suerbaum S, Michetti P (2002) *Helicobacter pylori* Infection. *N Engl J Med* **347**(15), 1175-1186. https://doi.org/10.1056/nejmra020542

Vilela SF, Barbosa JO, Rossoni RD, Santos JD, Prata MC, Anbinder AL, et al. (2015) *Lactobacillus acidophilus* ATCC 4356 inhibits biofilm formation by *C. albicans* and attenuates the experimental candidiasis in *Galleria mellonella*. *Virulence* **6**(1), 29-39. https://doi.org/10.4161/21505594.2014.981486

Wallace TC, Guarner F, Madsen K, Cabana MD, Gibson G, Hentges E, et al. (2011) Human gut microbiota and its relationship to health and disease. *Nutr Rev* **69**(7), 392-403. https://doi.org/10.1111/j.1753-4887.2011.00402.x

Williams NT (2010) Probiotics. *Am J Health Syst Pharm* **67**(6), 449–458. https://doi.org/10.2146/ajhp090168

Wu, W., Jin, Y., Bai, F., & Jin, S. (2015). *Pseudomonas aeruginosa*. *J Mol Med Microbiol* **2**, 753–767. https://doi.org/10.1016/b978-0-12-397169-2.00041-x

Wu Y, Hu S, Wu C, Gu F, Yang Y (2021) Probiotics: potential novel therapeutics against fungal infections. *Front Cell Infect Microbiol* https://doi.org/10.3389/fcimb.2021.793419.

List of abbreviations

B buffer	Breaking buffer
BSA	Bismuth Sulfite Agar
DRBC	Dichloran Rose Bengal Chloramphenicol
GIT	Gastrointestinal Tract
GRAS	Generally Regarded As Safe
HCCA	α-cyano-4-hydroxy-cinnamic acid
LAB	Lactic Acid Bacteria
MHA	Mueller Hinton Agar
mMRS	Modified de Man-Rogosa Sharpe agar
MRS	De Man, Rogosa and Sharpe
MRSA	Methicillin-Resistant *S. aureus*
MSSA	Methicillin-Sensitive *S. aureus*
O.G.Y.E.	Oxytetracycline Glucose Yeast Extract
OGY	Oxytetracycline Glucose Yeast
PCIA	Phenol Chloroform Isoamyl Alcohol
PDA	Potato Dextrose Agar
PQS	*Pseudomonas* Quinolone Signal
QPS	Qualified Presumption of Safety
QS	Quorum-sensing
RBC	Rose Bengal Chloramphenicol
SEM	Scanning Electron Microscopy
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WL	Walerstein Laboratory
YEPD	Yeast Extract Peptone Dextrose

DECLARATION OF ORIGINALITY

I Ana Gabrić declare that this master's thesis is an original result of my own work and it has been generated by me using no other resources than the ones listed in it.

Signature