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Mentor: Željka Maglica, PhD, Assistant Professor

SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
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Utjecaj polisaharida na probiotičke bakterije
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Summary

There are between 1000 and 1150 bacterial species that can be found in the human microbiome along with different species of viruses, fungi, protozoa and archaea. While inhabiting the distal small bowel, caecum and colon, their task is to metabolize substances in the lumen to provide an additional source of nutrients. Products that contain these commensal, nonpathogenic, microorganisms and that benefit the host are called probiotics. The most commonly used microorganism strains are the bacterial genera *Lactobacillus* and *Bifidobacterium,* as well as the probiotic yeast *Saccharomyces boulardii*. There is vast body of evidence documenting their beneficial effect to the host by modulating the host's immune system or by affecting other undesired microorganisms or their products.

However, like every other microorganism, bacteria and yeast require certain substances which would provide them with a suitable environment to grow. These substances are non-viable food components that confer health benefits to the host, and are also known as prebiotics. Prebiotic compounds are defined as carbohydrates or soluble dietary fibers such as inulin, β-glucans and fructooligosaccharides. Different prebiotics stimulate the growth of different bacterial species and it is important for them to reach the colon unchanged in order to stimulate the growth and activity of intestinal microbiota.

Many studies have shown a significant increase in number of probiotic bacteria in the presence of mushroom polysaccharides. Even edible mushrooms such as M. procera and S. crispa demonstrated high stimulation on the growth of lactobacilli and thus confirmed their potential use as nutraceuticals.

Key words: human microbiome, probiotics, *Lactobacillus*, *Bifidobacterium*, prebiotics, β-glucans, fungal polysaccharides

Sažetak

Postoji 1000 do 1150 vrsta bakterija koje sačinjavaju ljudski mikrobiom. Osim bakterija, ljudski organizam nastanjuju i virusi, gljivice, protozoe i arhee. Njihova uloga u tankom i debelom crijevu je metabolizam tvari iz lumena kako bi omogućili apsorpciju hranjivih tvari. Proizvodi koji sadrže ove ne-patogene, komenzalne mikroorganizme nazivaju se probiotici. Sojevi bakterija koji se najčešće upotrebljavaju zbog svog probiotičkog djelovanja su rodovi *Lactobacillus* i *Bifidobacterium* te kvasac *Saccharomyces boulardii*. Postoji mnoštvo dokaza za različita djelovanja probiotika. Smatra se da većina njihovih blagotvornih učinaka proizlazi iz kontrole ljudskog imunosnog sustava te uklanjanja patogena i njihovih štetnih nusprodukata.

Kao i svi mikroorganizmi, bakterije i kvasci zahtijevaju određene tvari koje će im osigurati pogodan okoliš za rast i razvoj. Takve supstance su neodrživi sastojci hrane koji ostvaruju blagotvorno djelovanje na zdravlje domaćina, poznatiji kao prebiotici. Kemijski spojevi koji čine prebiotike su ugljikohidrati ili topljiva prehrambena vlakna kao što su inulin, β-glukani i fruktooligosaharidi (FOS). Različiti prebiotici će poticati na rast različite vrste bakterija. Zbog toga je važno da do probavnog sustava dođu nepromijenjeni.

Mnoga istraživanja pokazala su znatno povećanje broja kolonija probiotičkih bakterija u prisutnosti polisaharida iz gljiva. Čak su i polisaharidi iz jestivih gljiva kao što su M. procera i S. crispa pozitivno stimulirali rast laktobacila te tako dokazali vlastiti potencijal za korištenje kao nutraceutici.

Ključne riječi: ljudski mikrobiom, probiotici, *Lactobacillus*, *Bifidobacterium*, prebiotici, β-glukani, polisaharidi iz gljiva

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1. Introduction

The human gut microbiota is composed of trillions different microorganisms from different species of bacteria, viruses, fungi, protozoa and archaea (1). Researchers have concluded that there are between 1000 and 1150 bacterial species, that can be found in the human microbiome. Each individual has at least 160 of those species in their gastrointestinal (GI) system (2). The distal small bowel, cecum, and colon have increased bacterial colonization relative to more proximal regions, with up to 10^{10} to 10^{12} colony-forming units (CFU) per gram of intestinal content in the colon (3). These commensal organisms in our intestines play an important role metabolizing substances in the lumen and in so doing provide an additional source of nutrients (4). They also may occur naturally in certain yogurts and fermented food. Commercial products where well-known commensal microorganisms can be found are called probiotics.

The word *"probiotic"* originates from Latin meaning *"for life"*, though there are also roots in Greek: *"bios"* meaning *"lively"* or *"fit for life"* (5). Although the positive effects of probiotic bacteria in fermented milk and dairy products were familiar even in the ancient Egypt (6), probiotics have been a subject of interest among the scientific community since the beginning of the twentieth century. Russian scientist and Nobel laureate Elie Metchnikoff first presented the thesis that the gut microflora can be modified, and harmful microbes replaced with beneficial ones. He proposed that intestinal autointoxication is caused by putrefactive or proteolytic bacteria that generate toxins in the large bowel and justified it by describing how proteolytic bacteria *Clostridia* produce toxic substances such as phenols and ammonia through the digestion of proteins in human bowels.

In 1917, during a shigellosis outbreak, the German professor Alfred Nissle isolated a strain of *Escherichia coli* from a soldier who was not affected by the disease and used it to treat people suffering from shigellosis and acute gastrointestinal salmonellosis. Henry Tissier of the Pasteur

Institute isolated another important probiotic bacterium called *Bifidobacterium,* which was initially used to treat diarrhea in babies (7). The name itself was first used in 1953 by the German Werner Kollath who suggested the term "probiotics" to describe different organic and inorganic substances related to health benefits.

Although the definition has changed through the years, it has been criticized because antibiotics could also be classified as substances, that contribute to intestinal microbial balance (6). That is why the current definition, as recommended by Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO), describes probiotics as viable, nonpathogenic microorganisms (bacteria or yeast) that are able to reach the intestines in sufficient numbers to confer benefit to the host (3). Benefits to the host can be numerous but ultimately it all comes down to the maintenance of a state of a complete physical, mental and social well-being and not merely the absence of disease and infirmity (8). Dietary changes, stress and consumption of antibiotics can disturb the balance of commensal bacteria (4) which can result in irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), diarrhea, constipation and even type two diabetes and eczema. Research suggests that probiotics may help manage all these conditions (3,9) by competitive exclusion and by the production of antibacterial agents known as bacteriocins. Another mechanism whereby probiotic bacteria may provide a health benefit is by modulating immune responses (4).

Commonly used bacterial probiotics include *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli* and *Streptococcus* species. *Lactococcus lactis* and some *Enterococcus* species have also been used. Most probiotic bacteria were originally isolated from healthy humans, since these were considered to be safe for human consumption, which means that probiotics have virtually no distinguishing characteristics relative to commensal organisms. As well as bacteria, probiotic yeast has also been

used. Currently the only probiotic yeast used is nonpathogenic *Saccharomyces boulardii* (3).

Like every other organism, bacteria and yeasts also require certain substances that will provide them with a suitable environment to grow and proliferate. These substances are called prebiotics and in 2007 FAO/WHO experts defined them as a nonviable food components that confer a health benefit on the host associated with modulation of the microbiota. Prebiotics may be used as an alternative to probiotics or as an additional support for them. An important feature is that different prebiotics will stimulate the growth of different indigenous gut bacteria. Some examples of artificially produced prebiotics are lactulose, galactooligosaccharides, fructooligosaccharides (FOS), maltooligosaccharides, cyclodextrins and lactosaccharose. Fructans, such as inulin and oligofructose, are believed to be the most used and effective of the to many species of probiotics (10). However, there is a vast body of evidence indicating that mushrooms demonstrate immunomodulating, antiviral, antidiabetic, antitumor, antioxidant, antibacterial and hypocholesterolemic effects. (10) That is why there have been studies about effects of fungal polysaccharides on probiotic bacteria. Some of the fungal polysaccharides extracted from 53 different mushrooms revealed stronger activity than inulin and FOS indicating that the development of nutraceuticals, which contain growth substrates from mushrooms, are necessary (11). Unfortunately, the precise mechanism of action of fungal polysaccharides is not yet fully understood, but there are a few suggested mechanisms of action that will be discussed in this work. An important criterion when selecting a probiotic strain is its ability to survive in the acidic conditions of the gastric tract. Substances that have been proven to have an effect on bacterial pH homeostasis are malt, wheat and barley (12). Correspondingly, considering the features of both probiotics and prebiotics, there is an important and rising market of synbiotics. These represent synergistic combination of both of these products and were

created in order to overcome possible difficulties in the survival of probiotics in the gastrointestinal tract (6, 10).

2. Purpose of the thesis

The aim of this study is to collect and analyse the effects and mechanisms of action of different polysaccharides on various probiotic bacteria. There are currently a few published studies directed towards understanding the precise impact of food supplements on beneficial microorganisms and critical literature analysis will help to clarify this topic.

3. Commonly used microorganism strains and their mechanism of action

The most common type of probiotic microorganisms are lactic acid bacteria (LAB, i.e. that convert sugars to lactic acid), such as *Lactobacilli*, *Lactococci* and *Bifidobacteria* (13). Their natural habitats are fermented food, especially dairy products and yoghurts, as well as the mucous membranes of humans and animals. They are generally recognized as safe and with no harmful side effects (6). In contrast, there are strains of nonLAB bacteria, such as *Bacillus*, *Propionibacterium* and *Escherichia coli*. They are also used as probiotics but are rarely included in dairy or other food commodities. However, non-LAB bacteria are usually applied as lyophilized or encapsulated pharmaceutical preparations (13). Recently there has been an increase in probiotics using yeasts. The only probiotic yeast used is the nonpathogenic *Saccharomyces boulardii*, mostly due to its resistance to antibiotics (3).

3.1 Genus *Lactobacillus*

Lactobacilli are fastidious, gram-positive bacteria that populate nutrient-rich habitats associated with food, feed, plants, invertebrate and vertebrate animals and humans. Moreover, this genus comprises more than 200 species characterized by a phylogenetic and metabolic diversity that exceeds that of a typical bacterial family. Due to their economic importance, their metabolism and genetics, have been extensively studied. These rodshaped, non-spore-forming bacteria differ in terms of host range, colonization site (gut, oral cavity, vagina) and the degree of specialization. Together with *Bifidobacterium,* they are one of the first commensal microorganisms that habituate human body upon birth and are mostly facultative anaerobic or microaerophilic bacteria that ferment carbohydrates (14).

The species for which a vertebrate-associated lifestyle is best understood are *L. reuteri, L. ruminis, L. salivarius, L. johnsonii, L. amylovorus* and *L. iners.* These species are usually found in the oral cavity and digestive tract of vertebrates, with the exception of *L. iners*. There are number of characteristics that reflect their adaptation to gastrointestinal conditions: they tolerate bile acids, are highly acid resistant and ferment oligo- and polysaccharides present in the diet of their hosts. In addition to digestion, they have been found to have an impact on immunity and brain activity. Furthermore, *Lactobacillus* species grow optimally at 37°C and higher, which is the body temperature of most mammals and birds (14, 6, 4).

The association of lactobacilli with invertebrates is a more recent discovery. Insect-associated species are distributed across the *Lactobacillus* phylogeny: clusters in the *L. kunkeei* and *L. mellifer* groups and in the *L. helsinborgensis* clade of the *L. delbrueckii* group. All four invertebrateassociated groups have small genomes and extremely limited carbohydrate fermentation capabilities, being essentially restricted to a "sucrose and maltose diet". Additionally, heterofermentative lactobacilli associated with bees are fructophilic, they lack alcohol dehydrogenase activity and depend on the availability of fructose as an electron acceptor (14).

The average size of lactobacilli is 0.5-1.2 micrometers by 1.0-10.0 micrometers. Their shape varies from long, slender rods to short, coryneform coccobacilli and are non-motile. Commonly used media for selective isolation are Universal Beer agar, MRS Agar and Broth.

Some of the most common lactobacilli species used in probiotics are *L. acidophilus, L. casei, L. paracasei, L. rhamnosus, L. plantarum* and *L. brevis* (15).

3.2 Genus *Bifidobacterium*

Bifidobacterium is a genus of gram-positive, mostly nonmotile, anaerobic bacteria which take form of pleomorphic, coryneform rods. They usually appear as clubbed or branching and may display bifurcated ends, while some species may occasionally exhibit swollen coccoid forms. The average size is approximately 0.5-1.3 by 1.5-8.0 micrometers. It is interesting that *Bifidobacterium* spp. are sometimes arranged in pairs, in "V" arrangements, in chains and in palisades of parallel cells or rosettes.

Bifidobacteria are ubiquitous inhabitants of the GI tract, vagina and mouth (*B.dentium*) of mammals, including humans. These organisms are usually considered non-pathogenic. However, they have been implicated in human infections including abscesses. *B. dentium* appears to have the highest pathogenic potential, as a result of isolation from human dental plaque and caries (15). Regardless, strains of the genus *Bifidobacterium* are often used as probiotic bacteria due to their variety of resistance mechanisms to bile salts, which is important since the beneficial effects of probiotic bacteria must be generated in the presence of this fluid (16). Several studies have investigated the potential of bifidobacteria to prevent and/or treat colorectal cancer. It was shown that *B. animalis* displays antimutagenic activity during growth in MRS broth thereby antagonizing the action of the carcinogen 2-amino-3-methylimidazo [4,5-f] quinolone. Additionally, they have also been used to treat various gastrointestinal disorders such as diarrhea; the administration of *B. longum* supp. *infantis* and *B. breve* K-110 resulted in inhibition of rotavirus which is the predominant cause of sporadic diarrhea in infants (17). They can also ameliorate eczema, food allergies and influence the level of cholesterol in the body (6).

Furthermore, *Bifidobacterium* species are key members of the infant gut microbiome, and some of them are capable of completely dominating the gut of the breastfed infant. That dominance is linked to consumption of

human milk oligosaccharide (HMO); Bifidobacterium ferments HMOs into lactate and acetate which reduces luminal pH and inhibits the growth of pathogens and other bacterial taxa in the gut. It has also been found that the number and species of the genus *Bifidobacterium* can alter with age: *B. bifidum, B. breve, B. infantis* and *B. longum* are mainly found in the infant flora while *B. adolescentis* and *B. catenulatum* constitute the adult microbiome (18).

Strains of *Bifidobacterium* that are the most important constituents of probiotics are: *B. infantis*, *B. adolescentis, B. animalis* spp. *lactis*, *B. animalis spp. animalis*, *B. bifidum*, *B. breve* and *B. longum* (15).

3.3 *Saccharomyces boulradii*

Saccharomyces boulardii is a non-pathogenic yeast discovered in 1923 by French microbiologist Henri Boulard in Indochina and it has been used as a probiotic in the prevention and treatment of gastrointestinal disorders ever since (19). Historically, it was thought to be a different *Saccharomyces* species, before genetic analysis classified *S. boulardii* as a strain of the *S. cerevisae* species. Therefore, the correct nomenclature should be *Saccharomyces* (genus) *cerevisiae* (species) *var boulardii* (strain). Even though genetically very close, there are differences, which might be related to the number of genes involved in protein synthesis and stress response. Consequently, *S. boulardii* exhibits a faster growth rate within the intestinal tract than *S. cerevisae* due to its increased temperature optimum and its higher acid resistance (20). As a yeast, it is distinct from bacterial probiotics, specifically because of its intrinsic resistance to antibiotic treatment. Furthermore, once in the GI tract, *S. boulardii* reaches the maximum concentration within two days and can easily adapt due to its ability to grow at 37°C (19).

The most used strain for research is called CNCM I-745 and it is produced by Biocodex Laboratiories (Gentilly, France). It is very well characterized by much preclinical and clinical data (20).

An increasing number of potential health benefits are being attributed to *S. boulardii.* There are several mechanisms of action that are directed against the host as well as pathogenic microorganisms. They include inhibition of the growth of bacteria and parasites, reduction of gut translocation of pathogens, suppression of host cell adherence, which interferes with bacterial colonization, and antitoxin effects such as inhibition of toxin receptor binding sites (21). Therefore, it is not surprising that probiotic supplements with *S. boulardii* also modulates the host's gastrointestinal immune system by inducing the release of immunoglobulins and cytokines in response to the yeast itself (20).

3.4. Probiotic mechanism of action

As was mentioned earlier, there is a vast body of evidence documenting probiotic bacteria and yeasts to have a beneficial effect to the host through their ability to modulate hosts immune system, affect other microorganisms directly or act on microbial products, host products or food components. The kind of effect a certain probiotic executes depends on its metabolic properties, the molecules presented at its surface or on the components secreted. The integral parts of the bacterial cell, such as its DNA or peptidoglycan, also might be of importance for its probiotic effectiveness. Additionally, individual combination of these properties in a certain probiotic strain determines its specific probiotic action and consequently its affective application for the prevention and treatment of a certain disease (22,23).

In order to understand the effects prebiotics and other food supplements/ ingredients have on probiotic bacteria, it is crucial to comprehend the mechanisms and cellular signaling of probiotic microbiome.

New discoveries are being made every day. However, many reported mechanisms of probiotic actions are the result of *in vitro* experiments and must still be confirmed by *in vivo* studies.

The effects of probiotics may be classified into three modes of action. (I) Probiotics might be able to modulate the host's defenses including the innate as well as adaptive immune system. This kind of effect prevents and treats infectious diseases and (chronic) inflammation of the digestive tract. (II) Probiotics also have a direct effect on other microorganisms, commensal and/or pathogenic ones, meaning they have a huge impact on microbial equilibrium in the gut. (III) Finally, probiotic signaling pathways and metabolic products may also affect pathogenic microbial products like toxins, as well as host products such as bile salts and food ingredients. The outcome of such actions usually results in inactivation of toxins and detoxification of the host. There are even claims for anti-cancer activity of probiotics. However, it is important to keep in mind that there seems not to be one probiotic strain exhibiting all three principles, at least not to the extent of the remedy for all mentioned kinds of diseases. That is why further studies need to be performed using genomic, proteomic, transcriptomic and other "-omic" methods.(23).

4. Prebiotics

4.1 Characteristics of prebiotics

By definition, prebiotics are "indigestible food ingredients that selectively promote the growth or activity of beneficial enteric bacteria or other beneficial microorganisms, thereby benefiting the host" (3). They can be either natural food components in plant-based foods or the result of synthetic production via enzymatic conversion of sugars. Prebiotic compounds are generally carbohydrate structures or soluble dietary fibers (polymers) which are selectively metabolized by microbes in and on the body. During this process, the proliferation of probiotic microorganisms occurs, which also improves the health of the host (25). However, many food components, especially many food oligosaccharides and polysaccharides (including dietary fiber), have been claimed to have prebiotic activity without due consideration to the criteria required. In reality, not all dietary carbohydrates are prebiotics and there are clear criteria that are established for classifying food ingredients as prebiotics (25). First of all, it is important for prebiotics to reach the colon and therefore they must be able to withstand the digestive processes that occur prior to, as well as persisting throughout, the large intestine. This kind of resistance includes the prebiotic resistance to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption. The second criteria which allows the classification of certain food ingredient as a prebiotic is selective fermentation by potentially beneficial bacteria in the colon. Furthermore, prebiotics must selectively stimulate growth and/or activity of intestinal microbiota which means that the substrate must be able to increase the growth of an organism relative to other organisms and relative to the growth on a non-prebiotic substrate, such as glucose. Given these points, carbohydrates are considered to have suitable prebiotic activity result if they are metabolized as well as glucose by probiotic strains and are selectively metabolized by probiotics and not by any other intestinal bacteria. Lastly,

prebiotic substances must be stable enough to withstand food processing, such as by heat, low pH and Maillard reaction. If the substance was degraded as a result into its component mono- and disaccharides, or else chemically altered, then it would no longer provide selective stimulation of beneficial microorganisms (27).

4.2 Current understanding of fiber

Fiber is defined differently throughout the world. Traditionally, it was described as chemical components such as cellulose, hemicellulose, pectin and lignin, which is the only noncarbohydrate component of fiber. In 2001 the Institute of Medicine (IOM) developed the following set of working definitions for fiber in food supply:

"*Dietary fiber* consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants." (28)

"*Functional fiber* consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans." (28)

These definitions refer to the diversity of nondigestible carbohydrates in the food supply and include plant, animal and manufactured fiber sources that exhibit beneficial physiological effects in humans. Soluble fibers were considered to have benefits on serum lipids while insoluble fibers were linked with laxation benefits. Moreover, foods that are high in fiber contain more than just fiber and these additional substances provide some of the protective health properties of fiber, such as viscosity and fermentability; fermentable fibers are the ones metabolized by colonic bacteria. Ultimately, soluble fibers have more protective health properties than insoluble ones, but on the other hand, not all soluble fibers are viscous (28). Since different fibers have different features, and therefore can affect an organism and beneficial bacteria in various ways, it is important to categorize them in a certain manner in order to understand which of them are mostly used as

prebiotics and why do they have such immense effect on probiotic bacteria (Table 1).

Table 1

Classification of fibers based on their characteristics of solubility, fermentability, viscosity and functionality.

Fibers	Classification
Dietary fiber	Lignin
	Cellulose
	B-glucans
	Hemicellulose Pectins
	Gums
	Resistant starch
Soluble fibers	B-glucans
	Gums
	Wheat dextrin
	Psyllium
	Pectin
	Inulin
Fermentable Fiber	Wheat dextrin
	Pectins
	B-glucans
	Guar gum

	Inulin
	Pectins
Viscous Fibers	B-glucans
	Some gums (e.g. guar gum)
	Psyllium
Functional fiber	Resistant dextrins
	Psyllium
	Fructooligosaccharides
	Polydextrose
	Isolated gums
	Isolated resistant starch
Insoluble Fibers	Ligninn
	Some pectins
	Cellulose
	Some hemicelluloses
Non- fermentable Fibers	Cellulose
	Lignin
Non-viscous Fibers	Polydextrose
	Inulin

4.3 Prebiotic dietary fiber sources

Many categories and compounds offer health benefits to consumers in a varying range of efficacies (29). Non-digestible carbohydrates, including di-, oligo- and polysaccharides, galactooligosaccharides (GOS) and FOS as well as inulin have been studied most extensively and are considered the best prebiotics.(11) Due to their chemical structure, nondigestible carbohydrates are neither absorbed in the upper GI tract nor hydrolyzed by human enzymes and thus fulfill all the criteria mentioned above as excellent prebiotic substrates for colonic bacteria (11).

4.3.1 Frucooligosaccharide- classes and effects

Fructooligosaccharides (FOS) are also known as oligofructose and oligofructan and are classified as prebiotic food ingredients owing to their non-digestibility, fermentation by intestinal microflora and selective stimulation of commensal human microbiota (30). Chemically, FOS are polymers with a degree of polymerization from 3 to 9, and they consist of a sucrose molecule which is elongated by a chain of fructosyl units. There are three different types of fructooligosaccharides graded according to their linkage patterns. First are inulin-FOS which consist of linear non-reducing chains with β-(2,1) linkages (e.g. 1-kestose (1^F-β-D-fructofuranosylsucrose)). Levan-FOS have a β-(2,6) linkage formed between fructose units (e.g. 6-kestose (6^F-β-D-fructofuranosylsucrose)) and neo-FOS molecules where the D-glucose moiety of sucrose is linked directly to a fructose unit through a β-(2,1) linkage (e.g. neo-kestose (6^Gβ-D-fructofuranosylsucrose)) (31). Fructans, such as inulin and oligofructose, are believed to be the most used and effective in relation to many species of probiotics (10).

Inulin and oligofructose are natural food ingredients present in more than 36000 plant species, but they are most commonly found and extracted

from chicory. The roots are harvested, sliced and washed and then, using a hot water diffusion process, the inulin is extracted, purified and dried. The finished product is inulin powder which contains glucose, fructose and sucrose. There is also another type of inulin called "high performance" (HP) inulin which is manufactured by removing the shorter-chain molecules, residual sugars and oligomers and thus provides twice the fat mimetic characteristics of standard inulin. Moreover, oligofructose is also derived from chicory in the same way as inulin, the only difference is a hydrolysis step after extraction. As mentioned above, inulin is a polydisperse β (2,1) fructan which means that the fructose units of fructose polymers and oligomers are each linked by β (2,1) bonds. These bonds are the unique aspect of the structure of inulin because these linkages prevent inulin from being digested like other carbohydrates and is also responsible for its fiber effects and diminished caloric value. Oligofructose, on the other hand is defined as fructose oligosaccharide containing 2-10 monosaccharide residues connected by glycosidic linkages. The one derived from chicory contains both fructose chains and fructose chains with terminal glucose units. Differing functional attributes of inulin and oligofructose occur because of differences in their chain length. Precisely because of its longer chains, inulin is less soluble than oligofructose, but both are non-digestible by human intestinal enzymes. When they enter the colon, they get fermented by colonic microflora which results in the production of shortchain fatty acids and lactate. (29)

5. Fungal polysaccharides and their effect on probiotic bacteria

Since there is a growing demand for prebiotics and new, relatively cheap and effective sources must be found, a lot of research has been made on mushroom polysaccharides. There is a vast body of evidence indicating that mushrooms have immunomodulating and antiviral/antibacterial effects which are the results of metabolically active constituents including polysaccharides.

As structural components, mushroom polysaccharides form cell walls can be divided into two major types: rigid fibrillars of chitin and glucans, which are more abundant. Glucans constitute of β-glucans with β-1,3 and β-1,6 linkages as well as α-glucans. β-glucans are polysaccharides of Dglucose monomers and are already implemented in food products such as in bread, dairy and cereals. Moreover, they can be used as fat substitutes in low-fat yoghurts (11, 32).

5.1 The metabolic pathway of β-glucan catabolism

Before presenting evidence about the positive effects on probiotic microorganisms, it is important to explain the metabolic pathway of βglucans. Lam et al. elucidated that pathway using *B.longum* spp. *infantis* as a probiotic species along with proteomic analysis, two-dimensional gel electrophoresis and RT-PCR for validation. The cultures were grown on βglucans derived from barley, seaweed and mushrooms. Ultimately, a model for catabolism of β-glucans in *B. infantis* was made (Figure 1).

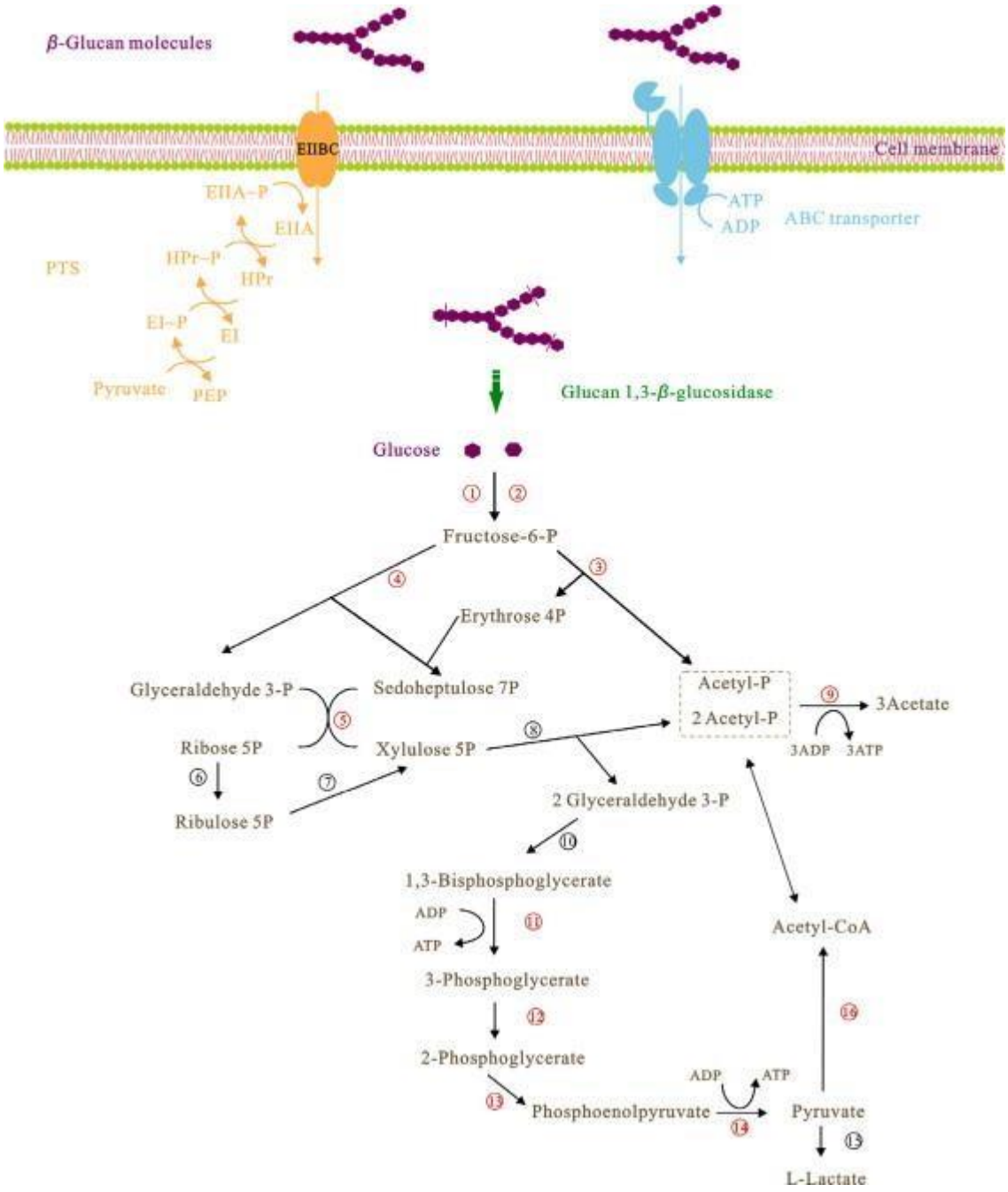

Figure 1 Model for catabolism of *β*-glucans in *B. infantis*. B-glucan molecules are transported from the media into the cell by ABC (ATP-binding cassette) transport system and PTS (phosphotransferase system) proteins. After the transport, the intracellular glucanase is separated to glucose by hydrolysis, and the glucose is subsequently incorporated into the central fermentative pathway "bifid shunt". The glycolysis separates in two ways; in anaerobic metabolism the pyruvate degrades and forms lactate while during aerobic metabolism acetyl-CoA is produced (30).

5.2 Studies regarding the effect of fungal and plant polysaccharides

The first study, made by R. Nowak et al., aimed to investigate the potential of polysaccharides from 53 wild-growing Polish mushrooms to stimulate the growth of *L. acidophilus* and *L. rhamnosus* as well as the digestibility of polysaccharide fractions (10). Mushroom polysaccharides were precipitated with ethanol from aqueous extracts. Determination of probiotic bacteria growth stimulation was made in U-shaped 96-plates, in an ELISA reader. Rogosa broth media without glucose was used and supplemented with 1.5% polysaccharide and inoculated with *L. acidophilus*, *L. rhamnosus* 1 or *L. rhamnosus* 2. The plates were incubated for 72 hours and the absorbance was measured using an ELISA reader. The negative control contained Rogosa broth without glucose, supplemented with polysaccharide and the positive control contained Rogosa broth with glucose and *Lactobacillus* strain. The results were presented as the percentage of lactobacilli growth in the presence of different polysaccharides by comparing them with the growth in the glucose-containing medium which was taken as 100%.

The most important step in the study was to determine the influence of mushroom polysaccharides on two *L. rhamnosus* clinical strains (*L. rhamnosus 1* and *2*) (10) isolated from human gastrointestinal tract which is presented in Figure 2.

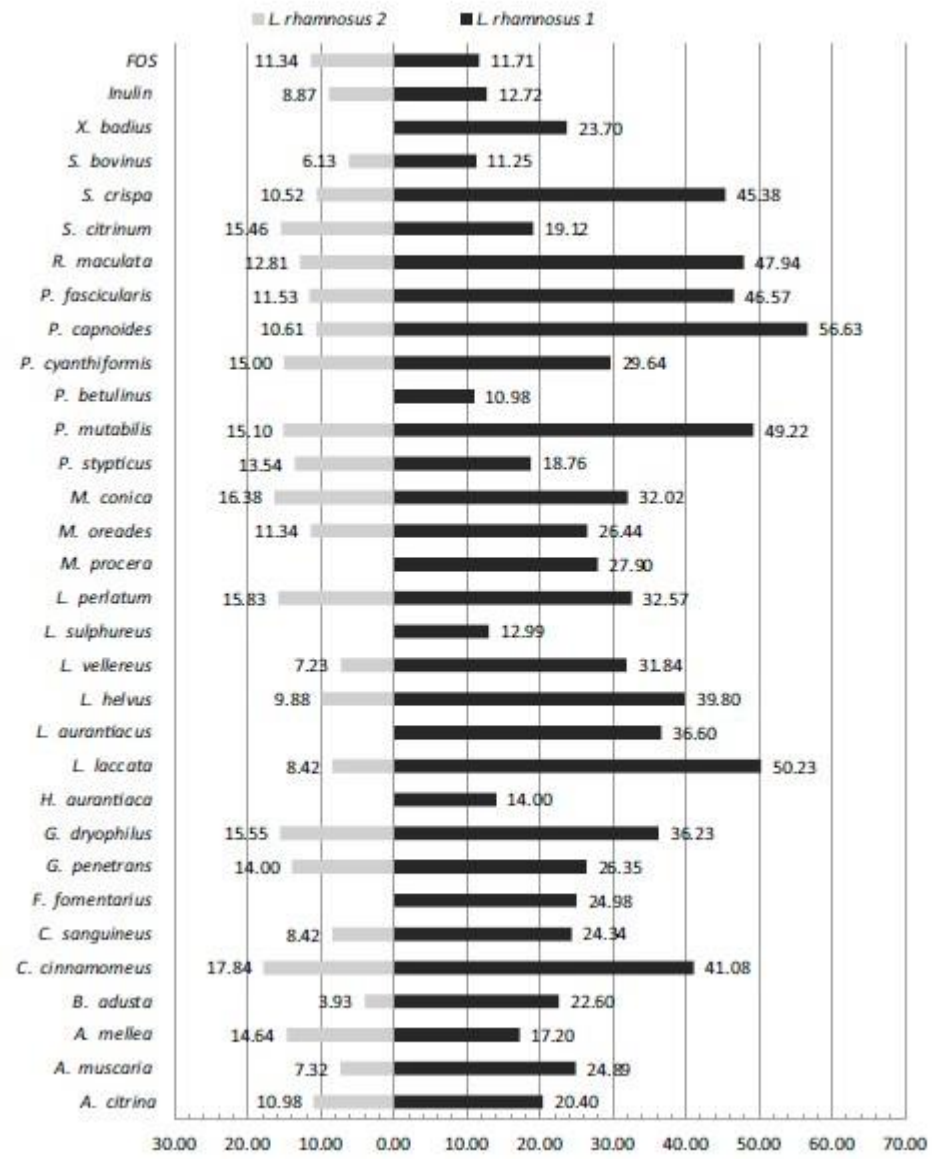

Figure 2 Growth promoting activity of mushroom polysaccharides with respect to L. rhamnosus 1 and 2 (%). The values calculated based on glucose activity (taken as 100%) for the species demonstrated statistical significance ($p \leq 0.05$) in Student's t test (10).

Of the 33 examples examined, only two species did not stimulate the growth of *L. rhamnosus* 1. Based on these findings, the authors divided the mushrooms into two grups; one group with the activities from 10 to 30% and the others with the activity above 30%. *P. capnoides* provided the highest activity (56.63%) while P. betulinus showed the worst activity with only 10.98%. Furthermore, polysaccharides from edible species *M. procera* and *S. crispa* demonstrated high stimulation on the growth of lactobacilli in which way they confirmed their potential using as nutraceuticals. FOS and

inulin showed similar activity, but both were significantly lower than those obtained from mushroom polysaccharides.

An important variable that was also evaluated was the resistance toward acid digestibility. This was tested by calculating the degree of hydrolysis of polysaccharides when they were subjected to artificial human gastric juice with both inulin and FOS as controls. The mushroom polysaccharides that were examined remained more than 90% undigested which suggests their capability to pass through the stomach unchanged and reach the colon and stimulate the growth of beneficial bacteria (10).

Another study was made in 2015, which evaluated the prebiotic properties of β-glucan and oligo-β-glucan from mushrooms (31). Chaikliang et al. performed *in vitro* fecal fermentation in anaerobic batch cultures using human fecal samples from three donors. The conditions simulated the ones that characterize human colon. Samples were taken at 0h, 24h and 48h to analyze the number of probiotic bacteria while being treated with β-glucans and oligo-β-glucans from mushrooms and commercial yeast β-glucan. Changes in the number of bacteria were analyzed by fluorescent *in situ* hybridization and short chain fatty acids (SCFA), which are produced at the end of β-glucans fermentation, were analyzed by HPLC. Moreover, a prebiotic index (PI) was calculated for the soluble β-glucans and oligo-βglucans to obtain a general quantitative measure of the prebiotic effect. It was calculated according an equation described in the paper (33) and represents a comparative relationship between beneficial and undesirable bacteria.

Ultimately, batch culture fermentation was used in order to monitor the effects of β-glucans and oligo-β-glucans on the fecal bacterial populations. After the samples were taken, results showed a significant increase in the levels of bifidobacteria and lactobacillus treated with βglucan from *Auricularia auricula* Judae after 24h of incubation (Table 2). On the other hand, Bacterioides and Clostridium species showed very low growth on the β-glucan from *Auricularia auricula* Judae and from *Schizophylum*

commune Fr which indicates that prebiotic effect of β-glucan is preferential and selective because it enhances the growth of bifidobacteria and reduces the growth of unwanted bacterial species such as bacterioides and clostridia. Furthermore, PI values at 48h of β-glucan from mushrooms were higher than the one from yeast and oligo-β-glucan. In contrast, acetate production, which was the most prevalent SCFA found in all samples, from oligo-β-glucan was higher than from β-glucan. Other SCFAs that were highly produced were propionate and butyrate; all of them represent great importance due to their hypocholesterolaemic effect. (31)

Table 2 Change in bacterial populations (log cell mL^{-1}) during fecal fermentation of βglucan and oligo-β-glucan in batch culture. (31)

Sample	Time (hour)	Bifidobacteria	Lactobacillus	Bacteroides	Clostridia	Eubacteria
β-glucan from Schizophylum commune Fr	0	10.34±1.24ab	10.91±1.01b	11.04±0.79b	11.07±1.25b	11.49±0.89a
	24	10.26±1.18^{b}	10.88±0.80^{ab}	11.28±1.03^{c}	11.15±1.23^{c}	11.51±0.98^{a}
	48	10.00±1.09^{a}	10.85±0.83^{a}	10.81±0.72^{a}	10.99±1.13^{a}	11.50±0.95^{a}
Oligo-β-glucan from *Schizophylum commune* Fr	0	9.96±1.01^{a}	10.59±0.91^{b}	10.95±1.14^{b}	11.00±1.09^{a}	11.18±0.73^{a}
	24	10.01±0.91^{a}	10.58±0.83^{ab}	11.09±1.27^{c}	11.10±1.28^{b}	11.51±1.16^{b}
	48	10.10±0.54^{a}	10.52±0.81^{a}	11.02±0.84^{a}	10.95±1.25^{a}	11.54±0.96^{c}
β-glucan from *Auricularia auricula* Judae	0	10.54±1.27^{a}	10.99±0.96^{a}	10.94±1.07^{b}	11.16±1.16^{c}	11.27±0.93^{a}
	24	10.59±1.30^{b}	11.00±0.97^{a}	10.74±0.63^{a}	11.04±1.07^{b}	11.38±0.88^{b}
	48	10.11±1.24^{b}	11.15±0.91^{b}	10.71±0.55^{a}	10.80±0.85^{a}	11.30±0.89^{a}
Oligo-β-glucan from *Auricularia auricula* Judae	0	9.64±0.79^{c}	10.78±0.55^{a}	11.00±1.07^{a}	11.01±1.04^{a}	11.34±1.00^{a}
	24	9.90±0.92^{a}	10.88±0.80^{b}	11.25±1.02^{b}	11.28±1.30^{b}	11.57±0.96^{b}
	48	10.37±0.88^{b}	10.81±0.73^{a}	11.43±1.25^{c}	11.22±1.25^{c}	11.57±1.23^{b}
Commercial yeast β-glucan	0	10.72±1.36^{a}	11.15±0.97^{b}	10.93±0.83^{b}	11.18±1.28^{a}	11.39±1.00^{b}
	24	10.70±1.22^{b}	11.23±1.00^{c}	10.96±0.97^{b}	11.35±1.30^{b}	11.49±1.00^{c}
	48	9.96±1.01^{b}	11.07±0.93^{a}	10.75±0.70^{a}	11.34±1.29^{b}	11.27±0.82^{a}

* Different letters in a column means are significantly different ($p < 0.05$)

6. Conclusion

Polysaccharides are polymeric carbohydrate molecules composed of long monosaccharide chains bound by glycosydic linkages. As prebiotics, they are selectively metabolized by commensal microorganisms such as lactobacilli, bifidobacteria and *S.cerevisiae.* Polysaccharides that are commonly used as prebiotics are FOS and inulin because they are effective compared to many other species of probiotics. However, recent data have indicated that mushroom polysaccharides such as β-glucans have preferential and stimulating effect towards probiotic bacteria. Moreover, they have demonstrated beneficial effects to human health, especially due to their immunostimulatory effects. Conversely, the molecular mechanism behind how polysaccharides, especially β-glucans, are being utilized by probiotic bacteria is still not fully understood. The mechanism of changes at a protein level that comes during the degradation of polysaccharides needs to be clarified in the future using "omics" and reverse genetics method. Furthermore, the hypothesis that the modification of the chemical structure of prebiotics alters their bioactivities should be thoroughly evaluated. Not only that it would improve the effect itself but also it would lead to understanding of the significance of their physical structure and chemical functional groups.

On the other hand, probiotic microorganisms have been used to treat a range of health conditions alongside polysaccharides. The mechanisms that caused beneficial health effects of described probiotics are not yet fully understood.

Overall, prebiotics, especially β-glucans extracted from mushrooms, have empowering effect on probiotic bacteria. Unfortunately, their mechanisms of action are not yet completely elucidated. Much more information is needed considering the definitive data on the species influenced by carbohydrates as well as structure-to-function information about the

carbohydrates themselves. Certainly, it will become possible to determine health applications and explain selective effects of both pro- and prebiotics in the light of new microbiological and biotechnological techniques.

7. Literature

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