Czech Univeristy of Life Sciences Prague

Faculty of Agrobiology, Food and Natural Resources

Department of Microbiology, Nutrition and Dietetics

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Growth of intestinal bacteria on lupine saccharides

Bachelor Thesis

Author: Viktorie Sedláčková

Supervisor: prof. Ing. Eva Vlková, Ph.D.
Advisor: Ing. Martina Geigerová

Declaration

I declare that the Bachelor Thesis “Growth of intestinal bacteria on lupine saccharides” is my own work that was conducted under a kind guidance of the supervisor and using available scientific literature sources, which are all listed in Bibliography.

In Prague ___________________________

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Growth of intestinal bacteria on lupine saccharides

Summary

It has been known, that the gastrointestinal microbiota of livestock is a complex and diverse ecosystem, which an animal's health is very dependent upon. One of the means of improving the composition of this microbiota in the gastrointestinal tract is application of probiotics, prebiotics or an appropriate combination of both: synbiotics. By means of subtle interventions, health benefits and an increase of some other livestock performance indicators can be achieved and thus the economic situation of farming can be improved.

Traditionally it has been soy that dominated as a protein source in animal feed. Due to its extensive use as well as due to ever-decreasing world's total area of non-genetically modified soy, many attempts have been made to find an appropriate protein source that would suffieciently substitue for it.

Lupine plant appears to have qualities to of a candidate substituent for soy in animal husbandry, because of itss high fibre and protein content, as well as due to its adaptability towards different climate and soil conditions.

This thesis' first part summarizes the theoretical background of the topic. In the second part, the growth of rabbit intestinal bacteria on lupine saccharides was investigated by means of cultivation using selective growth nutrients. Three varieties of lupine were investigated: Amiga, Dieta and Zulika. As a control nutrient, glucose was used. Main bacterial groups of interest were bifidobacteria, lactobacilli and E. coli.

Results obtained did show that bifidobacteria were stimulated by all growth nutrients. The most was stimulated variety Dieta, reporting concentration of 8.33 ± 1.68 log CFU.mL^{-1}, followed by variety Amiga with 8.19 ± 0.66 log CFU.mL^{-1} and variety Zulika which showed least significant growth, reporting 7.67 ± 1.29 log CFU.mL^{-1}. Lactobacilli did not show any significant growth on any of the nutrients used.

Keywords: lupine, prebiotics, oligosaccharide, rabbit, intestinal microbiota

Content

1 Introduction

One of many challenges animal husbandry has been facing in past years is assuring efficacy as well as quality while the demands on animal welfare had risen in the frame of European Union.

It has been known, that the gastrointestinal microbiota of livestock is a complex and diverse ecosystem, which an animal's health is very dependent upon. One of the means of improving the composition of this microbiota in the gastrointestinal tract is application of probiotics, prebiotics or an appropriate combination of both. Using subtle interventions can be achieved health benefits, increase of some indicators livestock performance and improving the economic situation of farming.

It has been a focus in animal husbandry to seek dietary sources of protein of an appropriate quality. Soybean meal has been dominating as a protein source in animal husbandry. Nevertheless, the world's total area of non-genetically modified soybeans has been gradually decreasing while the area of soy' transgenic varieties has been increasing. Due to such extensive use of soy, many attempts have been aimed on finding an appropriate protein substitute.

Lupine plant appears to have qualities to of a candidate substituent for soy in animal husbandry. Cultivation of so called "sweet" varieties of lupine has chased away past fear of alkaloid content in lupines and so due to its high fibre and protein content, as well as due to its adaptability towards climate and soil conditions, it would be a suitable feed component in the frame of the Czech Republic.

The thesis summarizes the theoretical background of the topic in its first part and in its second part there is a trial experiment on the growth of intestinal bacteria of a common rabbit on saccharides isolated from different varieties of lupine on described.

2 Literature overview

2.1 Microbiota of the mammalian gastrointestinal tract as a health asset

2.1.1 Introduction to gastrointestinal microbiota

Gastrointestinal tract is a complex system possessing very important functions, feed digestion and nutrients‘ uptake being of the most significance. It must be resistant and capable of performing good defence mechanisms, because it is exposed to external substances entering the system with food or already present as a part of intestinal microbiota (Stiburek, 2009). The gastrointestinal tract is a natural habitat for a large and diverse microbial ecosystem that is considered to be an “additional” living organ in mammals (Chaucheyras-Durand, 2009; Bunesova et al., 2015). Microbiome is a term describing all microorganisms living at a specific site – in this situation gastrointestinal tract (Fric, 2010). Intestinal microbiome is an important part of immune system defence: its products can affect proliferation of pathogenic species. Gastrointestinal wall represents a base of the defensive mechanisms against pathogens as it has a capability of acting against harmful substances. It can be said that commensal bacteria are an essential health asset with a nutritional function and a potentially positive effect on intestinal structure and homeostasis. The intestinal microbiota appears to have a protective function against infections, and to actively exchange developmental and regulatory signals with the host that primes and instructs mucosal immunity (Gaggia et al., 2010). Mucosal immunity functions as defence against microorganisms, as a barrier against food components or metabolites of present microbiota and immunity-regulating agent sustaining homeostasis which ensures management of damaged, mutated or non-suitable own cells (Wu et Wu, 2012).

It is believed that by enhancing the beneficial of the gut microbiotas it is possible to positively affect host well-being (O'Hara and Shanahan, 2007; Chaucheyras-Durand, 2009). Because in animal farming there is often a close proximity of individuals at farming sites, there are suitable conditions for host to host transmissions of microorganisms: it is therefore important to maintain a healthy indigenous microbiota as a barrier against pathogen infection (Gaggia et al., 2010).

Bacterial density and diversity of an individual is determined by physiological conditions of a host. Although the bacterial diversity among all mammals is considerable as it is influenced by mammalian phylogeny, phyla *Firmicutes* and *Bacteroidetes* usually dominate over mammalian intestine (Geigerova et al., 2014). The main microbial groups in monogastric mammals (such as rabbit, man, pig and chicken) are *Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Lactobacillus, Enterobacteriaceae, Streptococcus, Peptostreptococcus, Fusobacterium* and *Propionibacterium*. In polygastric animals (such as cow and sheep), the rumen is the most important microbial ecosystem with the predominance of fiber-degrading groups belonging to *Fibriobacter, Ruminococcus, Butyrivibrio* and *Bacteroides* together with major groups such as *Prevotella, Selenomonas, Streptococcus, Lactobacillus* and *Megasphaera* (Gaggia et al., 2010). Proportions of particular bacterial groups in gastrointestinal tract change in a lifetime of each mammal (Mueller et al., 2006; Geigerova et al., 2014). Diet is another factor influencing intestinal microbiota composition: while herbivores host wide variety of bacterial phyla, carnivores significantly less so and omnivores are at an intermediate level (Ley et al., 2008).

2.1.2 Gut microbiota: its evolution and composition

The evolution of gut microbiota of an individual possesses a coincidental character with consecutive phyla selection (Fric, 2009). The gastrointestinal tract of a new born mammal is at first colonised by commensal microbiota of its mother (Leahy et al., 2005). The composition of microbiota changes with age of an individual. In the beginning it is significantly influenced by the nature of birth itself – where and under what circumstances a new born was delivered, respectively. Later it is being influenced by a close surrounding: suckling mammals are exposed to microorganisms present in milk and faeces and on skin of a mother (Geigerova et al., 2014). Facultative anaerobic bacteria are first to colonize the intestine as there is oxygen present in gastrointestinal system right after the birth. Approximately 3 days after the birth anaerobic microbiota is established. Bifidobacteria dominate the intestinal tract of most suckling mammalians (Hopkins et al., 2001). After the suckling period is over, microbiota of an adult evolves in dependence on the character of its surrounding. Bacterial species dominate in adult mammals by population as well as metabolic activity. Phyla *Firmicutes* and *Bacteroidetes* were proven to prevail in mammalian intestinal microbiota. Although the mammalian gastrointestinal system is colonized by a few bacterial phyla only, diversity in genera and species is enormous. A particular species designates the composition of intestinal microbiota. Many species has been identified both in humans and animals, some of which were found only in individual species and are thus host-specific (Geigerova et al., 2014).

2.1.3 Intestinal microbiota: its main functions

Intestinal microbiota involves in various physiological processes in host body: microorganisms in the colon gain energy out of saccharides that were not fully digested in the small intestine. This is possible due to fermentation resulting in production of short-chain fatty acids (SCFA) and other substances. Production of acetic, propionic and butyric is most significant among SCFAs being produced and it positively affects metabolisms of glucosis and lipids in the liver. Presence of SCFAs lessens pH in the colon which arranges unsuitable environment for pathogenic bacteria (Den Besten et al., 2013). Furthermore, fermentation of non-digested saccharides improves absorption of calcium, magnesium, zinc and iron in the colon (Sholz-Ahrens et al., 2001). Intestinal microbiota is to a certain extent influential of the immune system’s development and the system appears to be continuously stimulated by it, too. Other functions of intestinal microbiota are sustaining integrity of the system, vitamins production, stimulation of mucous production and stimulation of intestinal angiogenesis.

The intestinal epithelium, together with the mucus, provides the defence by mediating the active sampling of commensal bacteria, pathogens and other antigens; three main types of immunosensory cells are involved: surface enterocytes, M cells and intestinal dendritic cells. Resident bacteria may exert a dual function, the stimulation of mucosal mechanisms of defence and the maintenance of the homeostasis of the immune response. Thus there is evidence of a correlation between the composition of the colonizing microbiota and variations in immunity (O'Hara and Shanahan, 2006). The finding of a connection between host well-being and its intestinal microbiota resulted among other in focus on searching for means of influencing intestinal microbiota in hosts favour. Several methods are used to achieve this aim. One of them is application of probiotics, prebiotics and synbiotics (Geigerova, 2014), that will be described further in following chapters.

2.1.4 Factors affecting intestinal microbiota

There are many factors intestinal microbiota is influenced by. Firstly it is the particular species to be considered, because internal environment as an important factor in dependent on it greatly. Composition of the intestinal microbiota is regulated by saliva, gastric acid and pepsin, bile containing bile salts and unconjugated bile acids, pancreatic juice (lipase) and intestinal motility. Regeneration of intestinal mucosal cells is also of a great importance (Jurankova, 2008).

Factors affecting microbiota are physiological, external and those connected with disease and its treatment. Microbiota may be severely influenced by disease and its treatment: long-term radiotherapy or declined pancreatic acid production after a surgical incident is one of the examples. Under such circumstances there are suitable conditions for coliform and potentially pathogenic bacteria (Jurankova, 2008). Physiological factors affecting intestinal microbiota are primary colonisation and age of an individual. As age keeps increasing, less saliva is secreted, swallowing is less frequent and renewal of mucosal cell is reduced. The main change in gut microbiota composition is nevertheless caused by the end of suckling period and transformation to an adult diet, where obligate anaerobes (Bacteroidetes, Prevotella, Ruminococcus) increase in number and the system tends to stabilize.

External factors include nutrition, living-conditions and environment, treatment and stress. Any changes in dietary habit or medication intake were proven to directly affect intestinal microflora. Another factor affecting intestinal microflora is stress. There has been a decline of lactobacilli observed in stress (Jurankova, 2008).

2.1.5 Site-specific intestinal microbiota

Lata et al. (2011) suggested that intestinal microflora contains 10x more cells than the whole organism and 100x more genes than there is present in the human genome. There are two main phyla among intestinal microbiota in mammals: *Bacteroidetes* and *Firmicutes.* Main genera would then be *Bacteroides, Clostridium, Fusobacterium, Eubacterium, Peptococcus, Bifidobacterium* and *Lactobacillus* (Stiburek, 2009). There is a significant difference between mono- and polygastric species when exact composition of the gastrointestinal microbiota is considered. Main bacterial groups present in monogastric animals, including rabbit, which are the focus of this work, are genera *Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Lactobacillus, Streptococcus, Fusobacterium, Peptostreptococcus, Propionbacterium* and bacteria of family *Enterobacteriaceae.* Bacterial diversity of an individual increases from carnivorous over omnivorous towards herbivorous species. Site-specific distribution of microorganisms in the gastrointestinal tract is designated by a host (Fuller, 1989).

There are both aerobic as well as anaerobic bacteria present in oral cavity. Most of them are feeding on residues of host food and considered to be of no significant harm, although some have showed to be assisting in breaking down the tooth enamel structure (Geigerova, 2014).

Stomach represents an aggressive environment due to extremely low pH and concentration of digestive enzymes. There are lactobacilli, streptococci and yeast as most significant microbial groups present in stomach. In comparison to other monogastric species, rodents‘ pH in stomach is not as low (3 – 5) which results in greater diversity in microbiota (Geigerova et al., 2014). Polygastric species unlike monogastric are characterized by rumen, an organ where fermentation activities occur significantly. Main bacterial phyla present in rumen of ruminants are *Bacteroides, Firmicutes, Proteobacteria, Actinobacteria* and *Tenericutes*. Other organisms present are protozoa, yeasts, bacteriophage and anaerobic fungi. Exact composition of the intestinal flora is highly variable among individuals (Geigerova et al., 2014). In the small intestine of both mono- and polygastric species, there are lactobacilli, streptococci, bifidobacteria, fusobacteria and coliform bacteria generally prevailing. The colon and caecum – a location of a notable microbial fermentation activity especially in monogastric species together with rectum – are characterized by *Bacteroides*, bifidobacteria, streptococci, eubacteria, fusobacteria, coliform bacteria, clostridia, lactobacilli, staphylococci, pseudomonades and yeasts (Svestka, 2007).

2.1.6 Gastrointestinal tract of rabbit and its specific features

Stomach of rabbit is an organ of sac-like appearance occupying 15% of the overall gastrointestinal tract volume. Rabbits cannot vomit feed already accepted into the system due to a well-developed cardia. Since pH in stomach is dependent on the precise site where it is measured (lowest values are spotted in the close distance from cardia), on the presence of cecotrophic faeces, on the age of a rabbit and on the time elapsed from feed intake, it varies between 5 – 6.5 up to 1 – 2 (De Blas et Wiseman, 2010).

Rabbit pups are equipped with a protective function of “milk oil” which provides an antimicrobial affect (Reesdavies et Reesdavies, 2003). Recent researches inform that except for breast milk, rabbit ingests also firm faeces being left behind by a mother within its first 21 days of life. It can be thus concluded that rabbit displays coprophagia. This behaviour however disappears with breast-feeding period. Coprophagic ingestion is important for establishing microbial community in the caecum and the large intestine, because as recent research discovers, such early implementation enhances the health state of individuals after breast-feeding period (Combes et al., 2014). As firm feed ingestion rises simultaneously with milk consumption decline, caudal sections of the tract are being colonised. Cecotrophy begins to evolve and is fully developed on about 30th day after the birth. This period is characterized by declining "milk oil" production and declining pH: defence against pathogens is in adult rabbit assured by lower pH (1 – 2). Balanced immunity in this temporary period is however dependant on how serene the transformation proceeds - rabbit intestinal tract is susceptible to intestinal infections in this phase of its lifecycle (Volek, 2015). The pH of stomach and small intestine in adult rabbits nevertheless varies from 1 to 2 and assures an almost sterility.

There are two types of chymus in rabbit stomach to be found: newly ingested feed and cecotrophic faeces stored in the bottom of the stomach which are in a practical sense content of caecum. Cecotrophic faeces rabbit collects right from the anus and because they are covered in mucin, they reach stomach after ingestion undisturbed (Reesdavies et Reesdavies, 2003).

Like in other monogastric species, the small intestine is a site where most important part of lipid, protein and starch digestion takes place. With pH value around 7 it represents location of passive and active nutrients transport, with most significant absorption and bile secretion. Main polysaccharide digested in the small intestine is starch with a final product being glucose that is relatively fast digested due to active transport (Reesdavies et Reesdavies, 2003). Main products of ongoing lipid hydrolysis are fatty acids and monoglycerides.

Rabbit liver produce daily at about 100 – 150 mL per 1 kg of body weight of bile. Main constituents of bile are bile acids, bile pigments bilirubin and biliverdin responsible for the yellow colour of bile. Lipids are emulgated by bile in order for them to be more easily digested as well as to improve absorption of lipophilic vitamins (Volek, 2015).

Rabbit caecum forms 49% of gastrointestinal tract and is defined by a weak muscle layer. Forming almost a half of the total tract volume, it represents a very important part of the tract (Volek, 2015). Age is a determinant when considering pH of caecum – it increases with age from 5.4 up to 6.8. Caecum is a major fibre digestion site. Non-starch polysaccharides and lignin are digested here. It has been known that polysaccharides of cell walls are hydrolysed by bacterial enzymes exclusively. Bacterial microflora together with caecotrophy allows enhancement of energy, amino acids and vitamins uptake. Main products of mentioned microbial degradation are volatile fatty acids – acetate, butyrate and propionate – that are effectively digested in caudal parts of the tract. In rabbits, the caecal metabolism of nutrients is similar to that of other herbivores, but the volatile fatty acids pattern exhibits a rabbit peculiarity, namely a predominance of acetate, followed by butyrate and then propionate (Gidenne et al., 2010). Indeed, with increasing age, the butyrate concentration increased while the propionate concentration decreased, leading to a propionate/butyrate ratio of <1 (Combes et al., 2011). The increase of the butyrate concentration may be addressed to an increase in some butyrate-producing bacteria that are distributed across several *Clostridium* clusters (Pryde et al., 2002). Significant changes within these clusters could have an influence on functional properties (such as butyrate production) which is supported by Combes et al., 2011 where at 70 days of age, the *Firmicutes* populations in rabbit caeca remained at high levels while *Bacteroides – Prevotella* decreased. Volatile fatty acids produced represent a regular form of energy source, acetate serves when higher fatty acids are synthesized propionate serves when glucogenesis and butyrate is utilised as an energy source in the liver.

Large intestine is a site with major cecotrophic activity. It proximal part is separated from its distal part by a thicker muscle layer - by so called *fusus coli*. This structure is exclusive for *Lagomorpha* order and it separates already fermented feed from non-digested fibre (Reesdavies et Reesdavies, 2003).

In rabbits, the composition of caecal bacterial community showed a dominance of strictly anaerobic bacteria, particularly non-sporulating Gram-negative bacilli and a scarcity or absence of *Lactobacillus, Streptococcus* and *Escherichia* (Gouet et Fonty, 1979; Combes et al., 2011). A high level of individuality in diversity of digestive microflora was nevertheless reported (Combes et al., 2011) in a similar way as previously suggested in pigs (Thompson et al., 2008). A progressive age-related change in the composition and apparent abundance of the species of the bacterial community took place as there was a progressive shift in the bacterial population observed from week to week (Combes et al., 2011). Indicated uncontrolled bacterial proliferation, demostrated by the high within-group distance between bacterial communities in the neonatal period, results into heterogenous collection of bacterial species (Tannock, 2005). The progressive reduction of the distances between the bacterial communities of two consecutive age groups from the neonatal period to the subadult period indicated a progressive development of stability with increasing age. At 70 days of age, the within-group distance decrease compared to previous stages and indicates a homogenization of the community's composition (Combes et al., 2011). Thus it can be suggested, that the 70-day-old caecal bacterial community in rabbit might have reached a steady state through a process of ecological succession. In rabbits, weaning is accompanied by a significant change in the bacterial composition and its metabolites (Konstantinov et al., 2006). Extending the milking period in young pups altered or delayed microbiota composition and behaviour compared with the control weaned rabbits (Combes et al., 2008). It has been suggested, that the beginning of solid feed intake promotes the establishment of the subadult digestive microflora and is likely to be an efficient way of managing the microbiota towards a better outcome for the host, for both health and digestive efficacy (Combes et al., 2010).

Cecotrophy is a specific character of rabbit gastrointestinal mechanisms. As it was mentioned, there are two types of excreta produced in this system: firm "buttons" and cecotrophic "soft" faeces. Soft faeces rabbits collect from their annus and they reach stomach undisturbed thanks to the film protecting them. Feed intake and firm faeces discharge take place simultaneously and it is alternated by discharge of cecotrophic faeces. The separation of firm and soft faeces takes places in caecum and proximal part of large intestine by mechanical separation of chymus. Cecotrophy advances protein ingestion and enhances natural rabbit diet poor in nitrogen. Enhancement is reached by reutilization of microbial enyzmes originating in caecum that are transported from caudal to the frontal parts of the digestive system (Volek, 2015).

2.2 Probiotics and prebiotics in animal husbandry

2.2.1 Probiotics in animal husbandry

2.2.1.1 Probiotics and their definitions

Probiotics are microorganisms roughly defined as those contributing beneficially to the host they live upon. Probiotic strains are generally bacterial species naturally occurring in the human or animal intestinal tract. Genera *Bifidobacterium* and *Lactobacillus* are nowadays the most frequently used probiotic agents. There has been a lot of beneficial effects on host' health addressed to probiotic bacteria so far.

The definition of "a probiotic agent" has been changing since the term was introduced. The first use of this term is often attributed to Lilly and Stillwell in 1965. In the very beginning it was used to describe "substances produced by one protozoan which stimulated another" (Lilly and Stillwell, 1965). Later was the term used for animal feed supplements which had a beneficial effect on the host by affecting its indigenous microbiota (Parker, 1974). Latter definitions describe as probiotic "organisms and substances which contribute to intestinal microbial balance". This definition was nevertheless including antibiotics and therefore was not satisfactory. Fuller revised it in 1989 to read "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989). This was further broadened by Haavenar and Huis in’t Veld as they described a probiotic as “a viable mono- or mixed culture of microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora” (Haavenar and Huis in’t Veld, 1992). In 1996 Conway suggested, that it was generally agreed on a probiotic being a preparation of live microorganisms which, applied to man or animal, beneficially affected the host by improving the properties of the indigenous microbiota. Todays most stated definition more or less corresponds to the one Fuller introduced in 1989: “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014).

The usage of probiotics nonetheless exists much longer than its definitions. In 1907 Ilja Mecnikov issued a study addressing the longevity of Bulgarian peasants to their regular consumption of fermented milk products containing live microbiological cultures. His theory on suppression of rotting processes is still considered one of the mechanisms of probiotic activity until today (Rada, 2011). Many sources mention Henry Tissier which isolated bifidobacteria for the first time in 1899. Isolation of the non-pathogenic strand of Escherichia coli in 1916 by Alfred Nissle is also considered by many to be a remarkable moment in the history of probiotics. This very strand is until nowadays used as a prevention of gastric infectious diseases.

2.2.1.2 Importance of probiotics in animal husbandry

Animal husbandry has been facing several challenges since the turn of 20th and 21st century to meet both food safety as well as efficacy. An example of such a challenge would be a ban on preventive application of antibiotics that has been progressively implemented in. That is why application of probiotics and prebiotics together with other methods assuring antimicrobial effects but not containing antibiotics has been focused on (Rada, 2005).

The importance of probiotics has a preventive character. They significantly involve in hosts‘ state of health as well as its development. Positive effects on mammalian younglings have been reported, among other lowering the risk of infection and diarrhoea (Nomoto, 2005). Moreover are probiotics efficient in maintaining balance in the intestinal microflora, compete with pathogens over epithelial adhesion, lessen the danger of pathogen proliferation and therefore also of disease manifestation. A vast amount of antibacterial substances are produced by probiotics and they are involved in nutrients‘ (short-chain fatty acids, amino acids, peptides) production, as well.

The very exact mechanisms by which probiotics affect the host are however largely unknown. It is largely believed that their effect rests upon the ability of stimulating both specific and non-specific immune system. Probiotics compete for pathogen binding and receptor sites as well as for available nutrients and other growth factors. They produce substances capable of inhibiting effect towards other organisms. Some probiotic agents even exert an ability of degrading toxin receptors at the intestinal epithelium (Nevoral, 2005). Implementing probiotics in animal husbandry aspires to higher resistivity towards infectious diseases, enhancing the growth performance, more efficient feed conversion ratio, feed digestion and nutrients‘ uptake and milk, meat or eggs quality and production (Fuller, 1997).

2.2.1.3 Most favoured genera for probiotic effects

Historically as well as nowadays, lactic acid bacteria have been favoured as probiotic microorganisms, genera *Lactobacillus, Streptococcus, Lactococcus* and *Enterococcus,* respectively. It is due to a long-term experience in processing of milk, production of sour pickled vegetables and use of silage. Additionally, mentioned genera are almost non-pathogenic and easy to manipulate with. An increasing demand on other criteria (growth abilities both in diet and chymus, epithelial adhesion, resistance towards intestinal acids or antagonistic ability towards other potentially harmful bacteria) in microbial colon manipulation has been nevertheless broadening the spectra of species used for the purpose. Genera *Bacillus, Bifidobacterium, Propionibacterium* or species *Clostrydium butyricum* would be examples of such microorganisms. Yeasts (*Saccharomyces cervisiae*) and moulds (*Aspergillus oryzae*) represent non-bacterial organisms used for probiotic treatment.

Specifically in animal husbandry, species favoured for probitic use are those of following genera: *Enterococcus, Lactobacillus, Pediococcus, Lactococcus*; eventually already mentioned *Saccharomyces cervisiae* and *Aspergillus oryzae* (Vlkova et al., 2013).

Genus *Bifidobacterium* has gained attention as a potential successful probiotic agent, because it is regularly found in the mammalian intestine and very rarely act as a pathogen. It was isolated from human and animal large intestine and oral cavity as well as from the large hindgut of honey-bees (Rada et Petr, 2002; Moran, 2015). Species *B. longum, B. bifidum, B. breve* and *B. adolescentis* have been commonly found in humans, whereas from animals a species *B. animalis* was often isolated (Rada, 2005). *B. animalis ssp. lactis* is often used in milk processing industry for its good technological qualities (Vlkova et al., 2004). Bifidobacteria are strictly anaerobic, which is a disadvantage when technology of the process is in question (Scardovi, cited in Rada 2005). Bacteria of genus *Bifidobacterium* form archetypal rods with ‘Y‘-shaped end and is gram-positive and non-sporulating. In mammalian sucklings, there is a typical high ratio of bifidobacteria present in the gastrointestinal tract that declines as an individual ages. Bifidobacteria provide host an antimicrobial effect due to ability of lowering pH in the gastrointestinal tract by producing strong acids (their derivatives, respectively), digestive enzymes and other substances (Bunesova et al., 2015).

Lactobacillus, genus of a lactic acid bacteria favoured for its probiotic effects, comprehends at about 150 species known. Only a few is used for the purpose of probiotic treatments, though. All species are defined by gram-positive non-sporulating rods that are rarely motile. Rods are arranged in short chains or palisades. Most of them are facultatively anaerobic and some of them are microaerophilic. They colonize gastrointestinal tract of humans and other mammals and birds. Greatest numbers of lactobacilli are found in the caudal part of the small intestine and ileum. Members of *Lactobacillus* genus rarely act as a pathogen (Bernardeau et al., 2008). Lactobacilli, *L. acidophilus* and *L. bulgaricus* most significantly so, produce hydrogen peroxide causing a decline of intraluminal pH. Bacteriocin reuterin produced by *L. reuterii* is known for its antimicrobial effects (McFarland, 2000).

An introduction of few other individual species with a significant use for probiotic purpose in livestock – *Enterococcus faecinum, Lactococcus lactis, Saccharomyces cervisiae* and an *E. coli* strain Nissle 1917 - will follow.

Enterococcus faecium is a facultative anaerobe commonly present in plant-based food and are formed by either pairs or chains of gram-positive cells. They exert positive effect on diarrhoea-related illnesses and acne (Mazankova et Kotaskova, 2011).

Yeast *Saccharomyces cervisiae* is non-pathogenic yeast and was reported to inhibit the growth of pathogenic organisms *in vitro* as well as *in vivo* (Mazankova et Kotaskova, 2011). It was first isolated by Henri Boulard in 1920. Thanks to its high resistivity towards substances in the gastrointestinal tract it is able to reach large intestine in a viable form and is therefore suitable for the probiotic purpose. It disposes of a stimulative effect on cellulolytic activity and is often administered in adult ruminants where other means of intestinal microbial management are needed since rumen-colonizing bacteria‘ application is problematic (Vlkova et al., 2013).

Lactococcus lactis was first introduced by Joseph Lister in 1878. It is a non-pathogenic gram-positive bacterium closely related to the genus *Streptococcus* and would be the most commonly used cheese starter. Moreover it is the most-precisely described lactic acid bacterium often used as a “model” when studying other lactic acid bacteria (Bolotin et al., 2001).

Probiotic strain Nissle of *E. coli* was introduced by Alfred Nissle in 1917 as a non-pathogenic strain able to inhibit growth of other pathogens (Lukas, 2003). Interestingly enough, it was isolated from faeces of a member of military during the First World War that did not develop in contrast to his companions’ infectious diarrhoea. It positively affects function of epithelial defence barrier due to epithelial cells‘ defenzine production (Sonnenborn et Schulze, 2009). The strain is applied also when treating some diseases, for example Crohn’s disease or chronic constipation. Moreover there has been a positive effect of *E. coli* Nissle 1917 on development of the gastrointestinal immune system in infants and inhibition of gut colonization by pathogen in infants and animals observed.

Another method of applying probiotics is application of multi-strain preparations. Number of strains may vary from two to several tens (Goren cited in Rada, 2005). “Nurmi concept” (Nurmi, 1992) – nowadays referred to as competitive exclusion principle – represents an extreme: it is a method of applying *per os* undefined intestinal content (usually content of caecum) of healthy adults to one-day-old chicks. Probiotics are often used in combination with other substances, such as prebiotics, enzymes, peptides, vitamins and even electrolytes (Rada, 2005).

2.2.1.4 Application of probiotics

Specific application of probiotics is decided according to many factors (species, age, diet and living conditions). The actual practical site of the application (application via feed, drinking water or individual type of application) must be considered, as well. As it was already mentioned, both single-strain and multi-strain treatments are available; this is therefore another aspect to be decided on (Rada, 2005).

Generally it can be stated that it is most convenient to use species that are present in the host naturally in high numbers. Thus in infants it is *Bifidobacterium* applied, whereas in young chicks and piglets *Lactobacillus* seems to be more effective. Probiotics can be applied to livestock in following forms (Fuller cited in Rada, 2005): as lyophilized powder, in drinking water, in form of a paste or as an aerosol. Especially common are applications with cultures as lyophilized powder.

Application of probiotics is particularly effective when applied on animal sucklings. Since their gastrointestinal tract is almost sterile, it is probable that a successful colonization by beneficial microbes will occur (Rada, 2005).

2.2.1.5 Bifidobacterium as a probiotic

Genus *Bifidobacterium* forms anaerobic gram-positive and non-sporulating rods often typically shaped into “Y” at one end, yet occurring in many different shapes and arrangements. Its representatives are found as a part of common microbiota of the digestive system as well as oral cavity of animals and humans. Moreover it was found in the gastrointestinal tract of bees (Rada, 2009). Specific species are variable according to the host: *Bifidobacterium magnum* (Scardovi et Zani, 1974) and *B. saeculare* (Biavati et al., 1991) were isolated from rabbit faeces exclusively, for a suitable example. Most notable species of the group would be *Bifidobacterium bifidum, B. adolescentis, B. infantis and B. longum* (Rada, 2009).

Antimicrobial effect of bifidobacteria is assured by production of digestive enzymes, B vitamins, acids (acetate and lactate, respectively) that cause intestinal pH to lessen. They represent an important factor in blocking activities of potentially harmful organisms. Moreover they exert positive effects on the systemic immune response; for example, on promotion of macrophages, stimulation of antibody production, and antitumor effects (Bornet et Brouns, 2002; Bunesova et al., 2015).

2.2.2 Prebiotics and synbiotics in animal husbandry

2.2.2.1 Introduction to prebiotics and synbiotics

In nutritional sciences there is much interest in dietary modulation of the digestive microbiota. There is essentially two main methods to be selected when positive management of digestive microflora is to be succeeded in. Next to *per os* application of live microbial cultures there is a method based upon supporting naturally present microflora by means of supplying it with substances stimulating its growth (Rada, 2005). These selective dietary substrates are commonly known as prebiotics and were introduced in 1995 precisely as: “non-digestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or more of limited number of bacteria in the colon that have the potential to improve health“ (Gibson et Roberfroid, 1995). Inulin, oligofructose, galactooligosaccharides and lactulose would be examples to such supplements (Rada, 2011). They enter the colon and serve as a substrate for the endogenous colonic bacteria, thus indirectly provide the host with energy, metabolic substrates and essential micronutrients. In other words, dietary modulation of the gut microflora by prebiotics is designed to improve health by stimulating numbers and/or activities of the bifidobacteria and lactobacilli (Manning and Gibson, 2004). For a dietary substrate to be classified as a prebiotic, at least four criteria are required: (1) the substrate must not be hydrolysed or absorbed in the stomach or the small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine, (3) consequently, be able of colonic flora alternation in favour of a healthier composition and (4) fermentation of the substrate should exert beneficial effects within the host (Gibson et Roberfroid, 1995). Most identified prebiotics are carbohydrates and oligosaccharides with different molecular structures regularly occurring in the human and animal diet. Dietary carbohydrates such as fibres, are candidate prebiotics, yet most promising are non-digestible oligosaccharides (NDOs). NDOs comprehend fructooligosaccharides (FOS, oligofructose and inulin) and galactooligosaccharides (GOS), lactulose, mannan-oligosaccharides, xylooligosaccharides, isomaltooligosaccharides, and raffinose-series oligosaccharides (raffinose, stachyose, verbascose). However a large number of other NDOs were also investigated (Gaggia et al., 2010). In humans, there have been positive effects when prebiotics were added to a diet shown; in animals though, the use of prebiotics, as an alternative to antimicrobial growth promoters, has shown contradictory results, while their role in the modulation of the gut microbial balance seems promising. They contribute to the establishment of a “healthier“ microbiota where bifidobacteria and/or lactobacilli become predominant and exert possible health-promoting effects at the account of potentially more harmful species (Gaggia et al., 2010).

Synbiotics are generally defined as a multi-componential mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract. The acquisition of data on the efficacy of synbiotic products as feed additives in livestock and poultry however needs further investigation, although there has been an increased focus on the topic in last decades (Gibson and Roberfroid, 1995; Gaggia et al., 2010).

2.2.2.2 Prebiotics: modes of action

Prebiotics beneficially interact with the physiology of animals by selectively stimulating favourable microbiota in the intestinal system. By doing so, their activities result in increased concentrations of short-chain fatty acids (SCFA), especially butyrate, which is the preferred energy source of colonocytes (Roediger, 1995) and the intestinal pH, which is associated with a suppression of pathogens and increased solubility of some of the nutrients. SCFA resorption may also modulate certain systemic physiological processes, such as glucose metabolism in the liver (Hesta et al., 2002). Furthermore butyric acid maintains integrity of epithel in the intestine and is a source of energy for cells regulating differentiation and cellular growth (MacFarland, 2009). Last but not least, prebiotic applications result in the competitive exclusion of pathogens by increasing numbers of microbiota that are associated with a healthy host. This microbiota can produce a variety of bacteriocins, which may also result in reduced pathogen numbers. Beneficial bacteria such as bifidobacteria also proved other beneficial effects as it was mentioned above (Bornet et Brouns, 2002).

2.2.2.3 Most favoured prebiotic supplements in animal husbandry

Fructooligosaccharides

Among the natural non-digestible oligosaccharides, fructooligosaccharides are those that meet all the criteria allowing classification as prebiotics (Gibson et Robberfroid, 1995). Chemically, they are defined as short- and medium-lenght chains of β-D fructans in which fructosyl units are bound by a β-galactosidic linkage. Some of their molecules have a glucose unit at its one end. Depending on the degree of polymerization (DP), it is either referred to as oligofructose (DP <9, average DP = 4.8) or as inulin (DP up to 60, average DP = 12). Inulin is prepared mostly by hot water extraction of chicory root, and oligofructose is obtained by partial enzymatic hydrolysis of inulin under strictly controlled conditions (Gibson and Roberfroid, 1995).

FOS are naturally found in plant-based food: especially then in above mentioned chickory, onion, garlic, asparagus, tomatoes and bananas. Typically, chicory fructan chains with DP below 10 are highly soluble in water, are rapidly fermented, and interact significantly in a selective manner with the intestinal microflora. Chains longer than DP 10 are slower fermented and hence arrive in more distal parts of the intestine and do not so explicitly affect the composition of the intestinal flora (Gibson et Roberfroid, 2008). Chickory inulin, as extracted from chicory roots, contains 30-50% chain with DP <10 and the remaining chains are longer. Oligofructose is composed of chain of DP <10 only. This is important in animal nutrition: according to the intestinal physiology of a host, which is defined by volume of the different compartments, feed transit time and the density of microbiota specifically in each compartment, or according to the organ intended as target (small intestine, cecum, or colon), either a short-chained oligofructose or long-chained inulin is to be decided for. The terms oligofructose and frucooligosaccharides are often used as synonyms, as the products they refer to are similar and the nutritional effects they exert are identical (Gibson et Roberfroid, 2008).

Dietary implementation of FOS including inulin has shown significantly beneficial effect on growth of probiotics both *in vitro* and *in vivo* conditions (Roberfroid, 2008).

Galactooligosaccharides

Galactooligosaccharides (GOS) are present naturally in human and bovine milk. Their chemical structure is glucose α1-4 [ß galactose 1-6]n, with n = 2-5. GOS have been shown to be readily utilized by bifidobacteria and lactobacilli; their prebiotic effects are supported by research conducted both on laboratory animals, including ‘human flora associated‘ brown rats, as well as on humans (Tuohy et al., 2005).

Transgalactooligosaccharides (TOS) – synthetically prepared GOS using lactose - are produced by ß-galactosidases and have transgalactosylation activity, which in simplified sense is a reaction involving the transfer of galactosyl groups into specific galactosyl acceptors (Kolida cited in Gibson et Roberfroid, 2008). After past evidence on its bifidogenic activities, TOS was concluded to be a suitable bifidobacteria-promoting substrate (Tuohy et al., 2005).

Raffinose-series oligosaccharides

Raffinose-series oligosaccharides (RSO) are characterized among other by the presence of galactose in most cases. They are naturally found in legumes and have a controversial position from the nutritional point of view, because it is often believed that they cause flatulence in humans. Yet there have been some positive effects on growth of bifidobacteria in the gut recorded. The group comprehends mainly rafinose (trisaccharide, α-DGal-(1-6)-α-D-Glu-(1-2)-ß-D-Fru), stachyose (tetrasaccharide, α-D-Gal-(1-6)-α-D-Gal-(1-6)-α-D-Glu-(1-2)-ß-D-Fru) and verbascose (pentasaccharide, α-D-Gal-(1-6)-α-D-Gal-(1-6)-α-D-Gal-(1-6)-α-D-Glu-(1-2)-ß-D-Fru) (Mitsuoka, 1992; Kohajdova et al., 2011).

Lactulose

Lactulose – chemically 4-O-ß-galactopyranosyl-D-fructose – is a disaccharide galactose-fructose isomerization product derived from lactose. It is traditionally used as a laxative in the treatment of constipation in humans. Although there is no sufficient scientific base for beneficial prebiotic effects on domestic animals yet, it has been shown to act as a prebiotic, as there are bifidogenic effects both in humans and laboratory animals (brown rats most significantly), reported (Tuohy et al., 2005).

2.2.2.4 Application of prebiotics in rabbit husbandry

In rabbit husbandry, the period around and just after weaning is critical in terms of digestive disorders. This issue is often linked with instabilities in the caecal microbiota and results mainly in clinical signs as loss of appetite, decreased growth, diarrhoea, and increased mortality. Facing this and some other challenges, there has been many researches on prebiotics in rabbit feed conducted. There has been a positive effect of applied fructooligosaccharides (FOS) on morbidity of rabbits described, for example (Morisse et al., 1993). In the experiment, rabbits were infected with *Escherichia coli* O103 and there have been significantly lesser clinical signs in the FOS fed group observed. There has also been a lower caecal pH, higher concentrations of caecal SCFA, and marked decrease in caecal ammonia observed. Volek et al. (2004) noticed an improved feed conversion in early-weaned rabbits fed 4% inulin. They also noted a lower mortality, a higher SCFA production, and a lower caecal pH in inulin fed rabbits, which seems to be corresponding with decreased caecal pH in 2% inulin fed rabbits described by Maertnes et al. (2004).

Lupine is economically an important plant (Gulewicz et al., 2008) and its seeds are worldwide used as a protein source in animal as well as human nutrition. Among nutritional value it is also appreciated due to its adaptability to different soil and climate conditions (TEN CLANEK).

Soya, mainly used for its protein nutritional character, has also been identified as an exclusive RSO source in animal husbandry (Grmanova et al., 2010).

also containing RSO - could be an alternative to soya both in the sense of protein value as well as prebiotic value.

3 Lupine in rabbit nutrition

3.1 *Lupinus:* botanic overview

Genus *Lupinus* – including over 200 species – represents a genus of flowering plants in the legume family, *Fabaceae*. There are both annual and perennial species included in the genus. Mostly it is annual species that are of an economic interest, namely *Lupinus albus L.*, *Lupinus luteus L.* and *Lupinus angustifolius*.

Most species dispose by a thick and deep-rooting conic root, where the nitrogen-fixing bacteria lay their tubers. Stem is upright, reaching from 50 up to 160 cm in height with different kinds of branching. Leaves are alternating and compound with 9-18 elongated oval leaflets, usually haired on one side. When flowering, lupine forms robust conic bunch-like arrangements with flowers of different kinds of colours. The fruit has a form of a flattened haired skin-like husk and seeds are carried in two separate chambers (Hybl et al., 2011).

Lupinus albus forms white flowers with a blueish spot. Even though it is primarily a self- pollinating crop, it is cross-pollinated from about 40%, as well. Compared to other species in the genera, it is quite sensitive towards the conditions and its vegetation period lasts 145-180 days. It would not tolerate temperatures below -3 °C (Hosnedl et al., 1998).

Unlike formerly described species, *Lupinus angustifolius* has a bold and poorly branched stem and its leaves are lightly haired. The flowers may be blue, pink or white. It requires a careful watering management, but is not sensitive towards amount of sunlight. Vegetation period lasts 120-135 days (Hosnedl et al., 1998).

Lupinus luteus, also known as European yellow lupine, has lightly haired leaves of light green colour. It forms yellow flowers with a black tip. The husk is densely haired with oval white seeds. Self-pollination prevails at this species and it would tolerate temperatures up to -2 °C (Hosnedl et al., 1998).

3.2 An insight into chemical composition of lupine

Lupine, with its approximate protein content of about 37%, generally contains about twice the amount of proteins than those found in commonly consumed legumes by humans. It is considered to be a good substitute candidate for soy. There is nevertheless a notable difference in the oil content between the two cultures: lupine seeds have about 10% of oil, whereas in soy the oil content reaches up to 20%. Furthermore, there is a nonnegligible amount of fibre present in lupine which enhances its dietary performance. Seeds of lupine, compared to other legumes, also contain a higher amount of soluble saccharides. Furthermore the seeds are considered to be a sufficient source of lysine. There are also insoluble polysaccharides and a subtle amount of starch present (Kohajdova et al., 2011). From what is known about oligosaccharide content, most distinctive is stachyose (2.8%), followed by sucrose (1.8%), raffinose (0.4%) and verbascose (0.3%), though the content of oligosaccharides varies according to the climate and soil conditions (Mohamed et Rayas-Duarte, 1995).

Dietary fiber - representing 40% of the kernel weight of sweet lupin – is present in higher levels than in most other legumes. The main component of the insoluble dietary fiber is cellulose (79%) (Smith et al., 2006).

Main storage proteins in lupine are globulins and albumins embodying approximately ¼ of the overall protein content (Salmanowicz, 2000). Lupine seeds are also considered to be a good source of lysine, although they are known for being poor in methionine, cysteine and threonine (Gulewicz et al., 2008). Compared to other legume plants, lupine shows usually lower amounts of potentially harmful substances (phytic acids, trypsin inhibitors, saponins and lektins, for example).

Even though lupines do not belong to the oil plants family, their seeds have considerable amount of oil. Lupine seed oil is characterized by a hospitable ratio of fatty acids: saturated fatty acids from 10% and unsaturated (oleic, linoleic and linonelic) from 90% of the fatty acids' content. Furthermore seeds contain phytochemicals with antioxidant activity such as polyphenols, primarily tannins and flavonoids. Lupine is relatively rich in minerals (potassium, magnesium, sodium, phosphorus, calcium, manganese) and vitamins (riboflavin, thiamine, vitamin C). Further there are carotenoids (lutein, zeaxanthin, β-carotene), tocopherols and other bioactive components (Kohajdova et al., 2011).

Lupines are among other characterized by a notable occurrence of quinolizidine alkaloids. These cause bitterness and toxicity of seeds and may exert negative effects on animals (Ruiz et Sotelo, 2001; Cook et al, 2012). That is why "sweet" lupine varieties with significantly low amounts of alkaloids are of great potential in animal as well as human diet (Wang et al., 2012).

3.3 Lupine in rabbit nutrition

It is in nowadays focus in the European conditions to keep seeking for dietary protein sources of reasonable quality. Traditionally it has been soy beans and soy products or soy meals, respectively, dominating in animal feed industry as a main component of animal feed. As a consequence of easily available and widely used soy meals, there has been a progressive decline in crops cultivated for protein content, especially legumes, in the conditions of the Czech Republic (Suchy and Strakova, 2015).

In the European Union, legumes occupy only about 2% of the arable land nowadays, while in the 1960s it used to be about up to 5%. It would be very hard to state that the overall situation of protein crops at the European market is satisfactory, because since 1960s, demand for protein components in animal feed has risen that - together with the fact that it is predominantly satisfied by soy beans or soy flour imported mainly from the USA and South America - puts the European protein crop market in a difficult position (Potmesilova, 2013). As a result, there has been an increased interest in Europe in finding new protein sources, and consequentially an increased interest in legume cultivation for animal feed as well as human diet (Suchy and Strakova, 2015).

Next to above mentioned need for alternative protein sources suiting domestic conditions well, there are other reasons for compensating for soy in rabbit feed mixtures. It has been reported that despite its beneficial effects on feed conversion ratio, soy meal increases the risk of digestive disruption when applied to rabbit feed in higher concentrations (Gutierrez et al., 2003; Volek et al., 2004; Volek and Marounek, 2009).

Lupine, a member of Fabaceae family, would be due to its relatively high protein and fibre content an advisable complement of both animal and human diet. There has been an increased interest worldwide in lupine cultivation. Today’s greatest producer of lupine seeds is Australia, where lupine products are commonly known and appreciated for their positive dietary effects (Suchy and Strakova, 2015). In the Czech Republic, cultivation of lupine has never been rampant, although its domestic environmental conditions are very suitable for lupine cultures. In past, there used to be certain scepticism towards lupine due to its alkaloids content. However, cultivation of sweet lupine varieties has administered decreased alkaloid content (below 0.005%) in lupine plants. These varieties have been commonly cultivated since the end of the 20th century (Houba et al., 2009).

There is a great difference in the nutrition content among the three groups of lupine varieties: Lupinusangustifolius – narrow-leafed lupine, Lupinusalbus – white lupine and

Lupinusluteus – yellow lupine (Petterson, 2000). The greatest protein content is in the seed of yellow lupine, where in some species it can be as high as 50%. White lupine has generally lower amount of protein compared to yellow lupine and narrow-leafed varieties has the lowest amount. Also the ratio of lipids varies among the three groups greatly. In white lupine, there may be up to 10% of oil in seed, whereas in other two groups it would be up to 5% maximally (Petterson, 2000; Volek et Marounek, 2011; Suchy et Strakova, 2015). Next to nitrogen-based components, there are both soluble and insoluble non-starch polysaccharides and raffinose-series oligosaccharides (Gdala, 1998). This may have a beneficial effect on rabbit digestive system (Volek et Marounek, 2009). Interestingly there is a low content of antinutritional components noted; lupine has a prominent position among other protein crops due to its trypsin inhibitor activity (Hybl et al., 2007).

White lupine varieties have showed sufficiently positive effect for fodder purposes in the Czech Republic with respect to their protein and lipids‘ content and their suitability for domestic soil and climatic conditions. Narrow-leafed varieties are also considered to be suitable, although less than white varieties. In the focus of rabbit husbandry, white lupine variety Amiga has been most worked with so far and has showed positive effects on digestive tract of rabbits and carcass meat quality while growth and feed conversion performances have not declined (Volek et Marounek, 2011; Volek et al., 2014). Moreover there has been an improvement in nutritional meat quality spotted by Volek et Marounek (2011) when there was an experimental diet with white lupine fed to rabbits. It is to be noted that beneficial effects were described when animals were fed ad libitum (Uhlirova et al., 2015). Volek et al. (2014) described an increase in milk production with lactating rabbit does fed diet with white lupine variety Amiga seeds added to feed. Next to an increased milk production, there has also been an increase in fat per kilogram of body mass production described, as well as a shift in fatty acids ratio in milk: notably lower amounts of saturated fatty acids and higher contents of oleic and eicosapentaenoic acids have been spotted. It can be deducted with respect to the scientific base of past researches, that white lupine is a suitable target as a source of crude protein in rabbit husbandry (Volek et al., 2004; Volek et Marounek, 2011; Volek et al., 2014).

Additionally, lupine can absorb a notable amount of pesticides and other potentially harmful substances and has an ability of enriching soil with nitrogen compounds. It is capable of enabling up to 200 kg of nitrogen per hectare in one harvesting season which is the reason for lupine often being cultivated for its soil-improving qualities. Among that, it is used for erosion control and pasture improvement. It can be thus stated that lupine, among its other beneficial properties, is an important green manure (Gulewicz et al., 2008; Kohajdova et al., 2011).

4 Experimental part

4.1 Hypothesis

Soy has been used as a primary protein source in animal husbandry. Among that, it has been found, that oligosaccharides present in soy exert a prebiotic effect when exposed to intestinal bacteria.

Many efforts have been put into finding a substitute for soy that would be both more suitable for the environmental conditions of the Czech Republic and at the same time it would have comparable or even better nutritional qualities. In accordance to that, utilization of oligosaccharides present in lupine by intestinal bacteria of a common rabbit was investigated in order to find out more about its nutritional benefits in addition to the protein content.

It was assumed, that lupine oligosaccharides should stimulate the growth of intestinal bacteria with probiotic properties (bifidobacteria and lactobacilli in this case) and should notbe utilized by E. coli.

4.2 Aims of the experiment

Sweet lupine varieties have been known to be rich in protein and therefore they have partially substituted for soy as a feed component in animal husbandry. Soy, among being rich in protein, also contains oligosaccharides which possess prebiotic effects when utilized by intestinal bacteria. A presumption of lupine disposing of similar oligosaccharides has been made; hence, an experiment that is a subject of this thesis, investigates utilization of oligosaccharides naturally present in lupine by intestinal bacteria of a common rabbit in order to determine if lupine can be an adequate substituent for soy not only from a protein content point of view, but also from a prebiotic qualities point of view.

5 Materials and methods

5.1 Growth of intestinal bacteria of common rabbit on lupine oligosaccharides

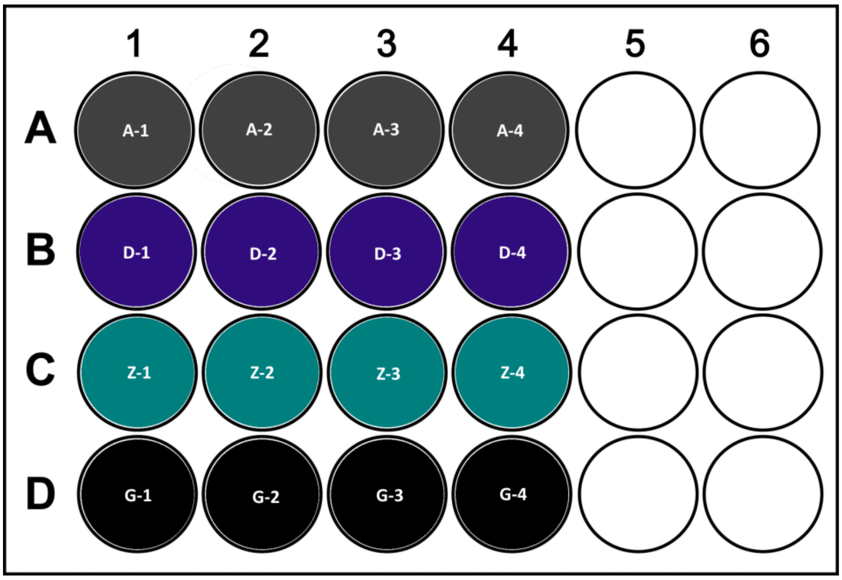
Oligosaccharides investigated during the experiment were isolated from 3 varieties of Lupinus albus: variety Amiga, Dieta and Zulika. Utilization of these oligosaccharides by relevant individual intestinal species of bacteria was then examined.

For the purpose of the experiment, 4 samples of the intestinal content of rabbit were used; 2 from the large intestine, 2 from caecum.

On the first day of the experiment, a microplate with stock solutions containing nutrients was prepared. Three different kinds of solutions were used: a) base solution containing lupine oligosaccharide that is being examined; in the experiment performed in the frame of this thesis, we investigated all three varieties of lupine separately, b) base solution, containing glucose and c) base solution alone as a control. All solutions were prepared as a 0.5% distilled water solution.

Layout of the microplate is shown in the following figure:

Figure 1: spacial arrangement of samples: RSO = sample containing raffinose series oligosaccharide; G = sample containing glucose; C = control sample



Each pit on the microplate was inoculated by 10 μL of each rabbit sample which formed the third dillution. The microplate was then incubated for 24 hours. All manipulation with the microplate was performed under strictly aseptic conditions.

After 24 hours, serial dilution was required in order to determine numbers of microorganisms. Serial dilution was in this case prepared up to the nineth dilution. Particular bacterial groups of interest were determined using selective growth media. Namely, lactobacilli were determined using Rogosa agar; TBX agar was used for determination of E. coli. Total viable count was examined using Wilkins-Chalgren agar and bifidobacteria were examined using the same growth media, but with an addition of muciprocin and acetic acid. Growth media was poured onto Petri dishes used for this purpose.

Particular serial dilutions were inoculated by 10 μL of incubated samples in the microplate from the previous day. 1 mL of each of serial dilutions were then applied on appropriate growth media and cultivated. Namely, lactobacilli were cultivated for 48 hours under microaerophilic conditions; the total viable count and bifidobacteria were cultivated for 48 hours in anaerobic conditions and E. coli for 24 hours in aerobic conditions.

Finally, the numbers of colonies were counted and evaluated.

6 Results

With accordance to the experiment, growth of intestinal bacteria was investigated using 4 samples taken from the intestines of a rabbit, 2 from caecum and 2 from the large intestine. There were 3 varieties of white lupine used for the purpose of the experiment: variety Amiga, Dieta and Zulika. Glucose was used in a reference nutrient sample.

Following table shows results of bacterial growth on lupine saccharides in comparison to the control samples and control growth nutrient medium:

nutrient	TC	Bifidobact.	Lactob.	E. coli
Amiga	9.75 ± 0.15	8.19 ± 0.66	< 3	9.62 ± 0.01
Dieta	9.81 ± 0.11	8.33 ± 1.68	< 3	9.48 ± 0.19
Zulika	9.87 ± 0.14	7.67 ± 1.29	< 3	9.61 ± 0.13
glucose	10.05 ± 0.10	6.10 ± 0.26	< 3	9.51 ± 0.05

TVC = total viable count

The analysis was done according to Scheffe’s test with the probability of $p=0.05$.

Bifidobacteria was stimulated when exposed to all of the four investigated nutrients. Variety Dieta had the highest concentration of 8.33 ± 1.68 log CFU.mL^{-1}, followed by variety Amiga - 8.19 ± 0.66 log CFU.mL^{-1}. Variety Zulika appeared to have stimulated bifidobacteria less sufficiently, reporting a concentration of 7.67 ± 1.29 log CFU.mL^{-1}.

E. coli as well was stimulated when exposed to all of the nutrients examined. Variety Amiga and Zulika had the highest numbers; 9.62 ± 0.01 log CFU.mL^{-1} and 9.61 ± 0.13 log CFU.mL^{-1}. Variety Dieta has shown the lowest concentration from all four investigated groups: 9.48 ± 0.19 log CFU.mL^{-1}.

There were no concentrations of lactobacilli of any significance in all of the investigated groups of samples.

After the statistical evaluation, there were are no significant differences among the groups of bacteria investigated, which may be due to the high value of Standard Deviation.

7 Discussion

The type and inclusion level of fibre, starch, and protein sources determine the quality of rabbit diets (Carabano et al., 2008). Soybean meal (SBM) has been the most widely used protein source in rabbit feeds. García-Ruiz et al. (2006), however, reported a higher mortality in rabbits fed a starter diet containing 129–170 g SBM per kg than in a diet containing 172–220 g sunflower meal (SFM) per kg. Higher mortality is one of many reasons why there has been an effort to implement substituents for soy in animal feed, for example an ever-shortening pool of non-GMO soy on the global scale or an ability of lupin seed oligosaccharides of reducing water absorption as demonstrated by Zduńczyk et al. 1998 on rats.

Recent research has focused on the ability of probiotic bacteria to ferment oligosaccharides, which are not hydrolyzed by the intestinal enzymes, and the selective utilization of oligosaccharides by bifidobacteria. Oligosaccharides have received much attention thanks to their prebiotic properties. The most studied prebiotics are inulin and fructo-oligosaccharides, but legumes are a good source of oligosaccharides known as a-galactosides or the raffinose family of oligosaccharides (RSOs), which are utilized by bifidobacteria (Gulewicz et al., 2002). Digestibility and fermentation of legume carbohydrates in the gut have both health and productive implications. Hence, a lot of research is aiming at of natural substances potentially able to exert prebiotic effects when ingested in the diet.

Lupins are legumes with one of the highest galactoside contents (7 to 15%) (Martinez-Villaluenga et al., 2004).

Research has shown that whole white lupine seeds (Lupinus albus cv. Amiga) are “a suitable dietary crude protein (CP) source for growing-fattening rabbits that can fully replace traditionally used protein sources (soybean meal and sunflower meal, mainly) without adverse effects on the digestibility of nutrients, caecal traits, performance or carcass parameters” (Volek and Marounek, 2009). Furthermore, Volek and Marounek (2011) reported that feeding rabbits a diet based on whole white lupin seeds (Lupinus albus cv. Amiga) affected the fatty acid profile of hind leg meat and perirenal fat in a favourable manner.

The importance of lupine (*Lupinus spp.*) as a valuable source of nutrients both in human or animal nutrition (Porres et al., 2006) has increased in recent decades. It is mainly thanks to its favourable content of protein, dietary fibre, minerals and fat as well as to its low content of some of the antinutritional components in so called sweet varieties. Another reason why lupine cultures are favoured is that they are able to grow under a broad spectrum of climate and soil conditions (Hill et al., 1977).

The experiment that was a subject of this thesis, investigated bacterial growth from the intestines of a common rabbit on lupine saccharides. Due to the fact that the results of studies on the nutritional effects of galactose-containing oligosaccharides from legumes have been quite inconsistent (Coon et al., 1990), a report on available literature sources, mainly done on other monogastric species, will follow.

White lupins dispose of high levels of non-starch polysaccharides, raffinose-series oligosaccharides and lipids and some varieties of WL seeds are low in alkaloids, protease inhibitor and other anti-nutritional factors (Martinez-Villaluenga et al., 2006).

Lupins are a rich source of RSOs that can be used as functional food ingredients. Martinez-Villaluenga et al. 2005 reported that when RSOs commonly found in legumes added to milk, produce products with the advantage of rapid growth and acidification rate and would likely increase the probiotic effect of the final functional product.

The effects of RSOs isolated from *Lupinus albus* var. Multolupa seeds on the growth and acid production of *Bifidobacterium lactis* Bb-12 cocultured with *L. acidophilus* in milk were investigated by Martinez-Villaluenga et al., 2006. Within this study, bifidobacteria had significant higher numbers of viable cells in milk with RSOs than in milk without RSOs during fermentation suggesting that the use of *B. lactis* Bb-12 and *L. acidophilus* in a mixed culture would have the advantages of rapid microbial growth and acidification rate. (Martinez-Villaluenga et al., 2005)

Rubbio et al., 2005 also reported promising results suggesting that lupin meals are suitable to substitute for soybean in Iberian pig feeds. In another study, in which Fritsch et al. 2015 investigated the microbial metabolism of four lactic acid bacteria during fermentation of different lupin substrates (sweet lupin flour, bitter lupin flour and lupin proteinisolate), the results showed that the secondary plant metabolites and potentially anti-nutritional substances in lupine flour did not significantly affect the fermentation performance if the bacteria tested.

In an experiment performed with broilers by Orda et al. (2006), increasing dietary levels of yellow lupine significantly decreased colony forming units Of E. coli and Lactobacillus.

In the study conducted on laying turkey hens, Zdunczyk et al. 2014 reported that in comparison with soybean, lupine seed exerted a higher content of neutral detergent fibre and RSOs as 20% dietary inclusion rate of lupine seeds contributed to an increase in total bacterial counts in the cecal digesta, which lead to positive changes in the population sizes of bacterial groups investigated. That would be in correspondence with findings of Lan et al. (2007) who reported that RSOs may promote competitive exclusion of potential pathogens in the intestines of young broiler chickens.

Smith et al., 2006 also revealed results in favour of lupine prebiotic properties as he reported significantly higher levels of *Bifidobacterium spp.* and significantly lower levels of the clostridia group on the lupine kernel fibre diet fed to healthy men in comparison with the control.

Oligosaccharides of raffinose series, which were subject of this thesis, are present in lupines in significant numbers; lupine showed 8,26 ± 0,14 g RSO for 100 g of lupine flour (Kodesova, 2017), which is comparable to the RSO content of soy (6,96 ± 0,21 g per 100 g of soy gristle).

In the experiment performed in this thesis, the growth of intestinal bacteria of a rabbit was examined in order to determine if bacteria with probiotic properties are stimulated by it or not. This was done by means of cultivating bacterial groups of interest on a growth media with selective nutrients. In comparison with the control nutrient (glucose), bifidobacteria were stimulated by lupine oligosaccharides more significantly. The most was stimulated variety Dieta, reporting concentration of 8.33 ± 1.68 log CFU.mL^{-1}, followed by variety Amiga with 8.19 ± 0.66 log CFU.mL^{-1} and variety Zulika which showed least significant growth, reporting 7.67 ± 1.29 log CFU.mL^{-1}.

As there is a limited number of studies dedicated to the topic, further experiments are suggested in order to accurate suggestions about prebiotic effects of lupins in rabbit diet that would eventually lead to broader implementation of lupins into rabbit feed in animal husbandry.

8 Conclusion

Research has shown that whole white lupine seeds are a suitable dietary crude protein source for growing-fattening rabbits that can fully replace traditionally used protein sources, without adverse effects on the digestibility of nutrients, caecal traits, performance, or carcass parameters. Furthermore, lupine seed in feed are most probably of a prebiotic nature, thus contributing to the intestinal balance of animals fed the diet containing them.

However, further investigations would be necessary in order to describe prebiotic properties of lupins with more precision.

9 List of bibliography

Aachary, A. A.; Prapulla, S. G. Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. Comprehensive Reviews in Food Science and Food Safety, 2011, 10.1: 2-16.

Amara, A. A.; Shibl, A. Role of Probiotics in health improvement, infection control and disease treatment and management. Saudi Pharmaceutical Journal, 2015, 23.2: 107-114.

Anjum, M. I., et al. Effect of dietary supplementation of multi-strain probiotic on broiler growth performance. Pak. Vet. J, 2005, 25.1: 25-29.

Bernardeau, M., et al. Safety assessment of dairy microorganisms: the Lactobacillus genus. International journal of food microbiology, 2008, 126.3: 278-285.

Biavati, B.; Mattarelli, P.; Crociani, F. Bifidobacterium saeculare: a new species isolated from feces of rabbit. Systematic and applied microbiology, 1991, 14.4: 389-392.

Bourdichon, F., et al. Food fermentations: microorganisms with technological beneficial use. International journal of food microbiology, 2012, 154.3: 87-97.

Bolotin, A., et al. The complete genome sequence of the lactic acid bacterium Lactococcus lactis ssp. lactis IL1403. Genome research, 2001, 11.5: 731-753.

Bunešová, V.; Geigerová, M.; Vlková, E. Bifidobakterie jako možná probiotika pro mláďata přežvýkavců. Veterinářství, 2015, 65 (7): 528 – 532

Carabano, R., et al. New concepts and objectives for protein-amino acid nutrition in rabbits. In: Proceedings of the 9th World Rabbit Congress, Verona. Fondazione Iniziative Zooprofilattiche e Zootecniche, Brescia, Italy. 2008. p. 135-155.

Carabano, R., et al. 1 The Digestive System of the Rabbit. Nutrition of the Rabbit, 2010, 1

Chaucheyras-Durand, F.; Durand, H. Probiotics in animal nutrition and health. Beneficial microbes, 2009, 1.1: 3-9.

Combes, S., et al. Coprophagous behavior of rabbit pups affects implantation of cecal microbiota and health status. Journal of animal science, 2014, 92.2: 652-665.

Conway, P. L. Selection criteria for probiotic microorganisms. Asia Pacific Journal of Clinical Nutrition, 1996, 5: 10-14.

Cook, D. et al. Alkaloid Profiling as an Approach to Differentiate Lupinus garfieldensis, Lupinus sabinianus and Lupinus sericeus. Phytochemical analysis, 2012, 23.3: 278-284.

Coon, C. N., et al. Effect of oligosaccharide-free soybean meal on true metabolizable energy and fiber digestion in adult roosters. Poultry Science, 1990, 69.5: 787-793.

Costa, F. G. P., et al. Economic and environmental impact of using exogenous enzymes on poultry feeding. Int. J. Poult. Sci, 2008, 7: 311-314.

Davies, R. R.; Davies, J. AE R. Rabbit gastrointestinal physiology. Veterinary Clinics of North America: Exotic Animal Practice, 2003, 6.1: 139-153.

De Blas J. C.; Wiseman J. (ed). Nutrition of the Rabbit (2nd edition). 2010. CAB International, Wallingford, UK, 66-82.

Den Besten, G., et al. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of lipid research, 2013, 54.9: 2325-2340.Dibner, J.J., Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Science 84, 634–643.

Fritsch, C.; Vogel, R. F.; Toelstede, S. Fermentation performance of lactic acid bacteria in different lupin substrates—influence and degradation ability of antinutritives and secondary plant metabolites. Journal of applied microbiology, 2015, 119.4: 1075-1088.

Frič, P. Střevní mikroflóra, gastrointestinální ekosystém a probiotika. Med Pro Praxi, 2010, 7: 408-414./ FULLER, Ray. A review. J. appl. Bacteriol, 1989, 66: 365-378..

Gaggia, F.; Mattarelli, P.; Biavati, B..Probiotics and prebiotics in animal feeding for safe food production. International journal of food microbiology, 2010, 141: S15-S28.

Garcia-Ruiz, A. I., et al. Effect of protein source and enzyme supplementation on ileal protein digestibility and fattening performance in rabbits. Spanish Journal of Agricultural Research, 2006, 4.4: 297-303.

Geigerová, M.; Vlková, E.; Skřiváňová, E.; Bunešová, V. Odlišnost v mikrobiotě trávicího traktu různých druhů savců. Veterinářství, 2014, 64 (7): 522 - 526

Gdala, J. Composition, properties, and nutritive value of dietary fibre of legume seeds. A review. Journal of Animal and Feed Sciences, 1998, 7: 131-149.

Gibson, G. R.; Robberfroid, M. B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. nutr, 1995, 125: 1401-1412.

Gibson, G. R.; Robberfroid, M. (ed.). Handbook of prebiotics. CRC Press, 2008.

Gibson, G. R., et al. Dietary prebiotics: current status and new definition. Food Sci Technol Bull Funct Foods, 2010, 7: 1-19.

Gouet, P. H.; Fonty, G. Changes in the digestive microflora of holoxenic* rabbits from birth until adulthood. In: Annales de Biologie Animale Biochimie Biophysique. EDP Sciences, 1979. p. 553-566.

Grmanová, M., et al. Naturally occurring prebiotic oligosaccharides in poultry feed mixtures. Folia microbiologica, 2010, 55.4: 326-328.

Gulewicz, P., et al. Biological activity of α-galactoside preparations from Lupinus angustifolius L. and Pisum sativum L. seeds. Journal of agricultural and food chemistry, 2002, 50.2: 384-389.

Gulewicz, P., et al. Effect of germination on the protein fraction composition of different lupin seeds. Food Chemistry, 2008, 107.2: 830-844.

Gutierrez, I., et al. Effect of protein source on digestion and growth performance of early-weaned rabbits. Animal Research, 2003, 52.5: 461-471.

Hill, C., et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol*, 2014, 11: 506-514.

Hlívák, P., et al. One-year application of probiotic strain Enterococcus faecium M-74 decreases serum cholesterol levels. Bratisl Lek Listy, 2005, 106.2: 67-72.

Hopkins, M. J.; Sharp, R.; MacFarlaine, G. T. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut, 2001, 48.2: 198-205.

Houba, M.; Hochmann, M.; Hosnedl, V. Luskoviny: pěstování a užití. 1. vyd. České Budějovice: Kurent, 2009. 44 s. ISBN 978-80-87111-19-2.

Hosnedl V.; Vašák, J.; Mečiar L. (1998). Rostlinná výroba - II (Luskoviny, olejniny). Agronomická fakulta ČZU v Praze, Praha, pp. 180.

Hýbl, M.; Ondřej, M.; Mezlík, T, 2007, Vhodnost lupiny pro různé pěstitelské podmínky ČR, Farmář 4/2007, 22-26

Hýbl, M.; Ondřej, M.; Seidenglanz, M.; Vaculík, A. Metodika pěstování lupiny bílé, žluté a úzkolisté: certifikovaná metodika. 1. vyd. Šumperk: Agritec, 2011. 32 s. ISBN 978-80-87360-02-6.

Jeffrey, J. S. Use of competitive exclusion products for poultry. University of California, 1999.

Juránková, J., et al. Intestinal microflora part I–general overview. Folia Gastroenterol Hepatol, 2008, 6.4.

Kodešová, T. Lupina jako zdroj prebiotických oligosacharidů. Bakalářská práce. Česká zěmědělská univerzita v Praze, 2017.

Kohajdová, Z. et al. Lupin composition and possible use in bakery–a review. Czech J. Food Sci, 2011, 29.3: 203-211.

Konstantinov, S. R., et al. Post-natal development of the porcine microbiota composition and activities. Environmental Microbiology, 2006, 8.7: 1191-1199.

Lan, Y., et al. Soy oligosaccharides in vitro fermentation characteristics and its effect on caecal microorganisms of young broiler chickens. Animal feed science and technology, 2007, 133.3: 286-297.

Lata, J.; Juránková, J. Střevní mikroflóra, slizniční bariéra a probiotika u některých interních chorob. Interní Med, 2011, 13.2: 63-69.

Lilly, D. M.; Stillwell, R. H. Probiotics: growth-promoting factors produced by microorganisms. Science, 1965, 147.3659: 747-748.

Leahy, S. C., et al. Getting better with bifidobacteria. Journal of Applied Microbiology, 2005, 98.6: 1303-1315. Ley, Ruth E., et al. "Evolution of mammals and their gut microbes." Science 320.5883 (2008): 1647-1651.

Lukáš, M.: Escherichia coli (Escherichia coli kmen Nissle 1917, sérotyp O6:K5:H1) jako probiotikum v klinické praxi. Remedia, 2003, 4: Aktuality

Martinez-Villaluenga, C. et al. Raffinose family of oligosaccharides from lupin seeds as prebiotics: application in dairy products. Journal of food protection, 2005, 68.6: 1246-1252.

Martinez-Villaluenga, C.; Frias, J.; Vidal-Varverde, C. Raffinose family oligosaccharides and sucrose contents in 13 Spanish lupin cultivars. Food chemistry, 2005, 91.4: 645-649.

Martinez-Villaluenga, C.; Frias, J.; Vidal-Varverde, C. Functional lupin seeds (Lupinus albus L. and Lupinus luteus L.) after extraction of α-galactosides. Food Chemistry, 2006, 98.2: 291-299.

Mazánková, D.; Kotásková, S. Probiotika z pohledu praktického lékaře--klinické indikace pro podání probiotik, výsledky dotazníkového šetření mezi praktickými lékaři. General Practitioner/Prakticky Lekar, 2011, 91.11.

McFarland, L. V. Normal flora: diversity and functions. Microbial ecology in health and disease, 2009

Mitsuoka, E. T. The human gastrointestinal tract. In: *The Lactic Acid Bacteria Volume 1*. Springer US, 1992. p. 69-114

Mohamed, A. A.; Rayas-Duarte, P. Composition of Lupinus albus. Cereal Chemistry, 1995, 72.6: 643-647.

Moran, N. A. Genomics of the honey bee microbiome. Current opinion in insect science, 2015, 10: 22-28.

Morrise, J. P., et al. Assessment of the activity of a fructo-oligosaccharide on different caecal parameters in rabbits experimentally infected with E. coli 0.103. *Ann. Zootech*, 1993, 42: 81-87.

Mourao, J. L.; Alves, A.; Pinheiro, V. M. Effects of fructo-oligosaccharides on performances of growing rabbits. *CECAV-Centro de Ciência Animal e Veterinária*, 2004.

Mueller, S., et al. "Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study." Applied and environmental microbiology 72.2 (2006): 1027-1033.

Nevoral, J. Prebiotika, probiotika a symbiotika. Pediatrie pro praxi. 2005, roč. 2005, č. 2, s. 59-65

Nomoto, K. Prevention of infections by probiotics. Journal of bioscience and bioengineering, 2005, 100.6: 583-592.

Nurmi, E.; Nuotio, L.; Schneitz, C. The competitive exclusion concept: development and future. International journal of food microbiology, 1992, 15.3: 237-240.

O'Hara, A. M.; Shanahan, F. The gut flora as a forgotten organ. EMBO reports, 2006, 7.7: 688-693.

O'Hara, A. M.; Shanahan, F. Gut microbiota: mining for therapeutic potential. Clinical Gastroenterology and Hepatology, 2007, 5.3: 274-284.

Orda, J., et al. Effects of increased dietary inclusion of yellow lupins and enzyme supplementation on performance, ileal digestibility of nutrients and microbial status of large intestine in broiler chickens. Archiv fur Geflugelkunde, 2006, 70.1: 14-21.

Parker, R. B. Probiotics, the other half of the antibiotic story. Anim Nutr Health, 1974, 29.29: 4-8.

Petterson, D. S., et al. The use of lupins in feeding systems-review. Asian-Australasian Journal of Animal Sciences, 2000, 13.6: 861-882.

Potměšilová, J. 2013. Postavení a možnosti využití lupiny v České republice, Evropské unii a ve světě. Krmivářství, 5, 38-43

Porres, J. M., et al. Nutritional evaluation of protein, phosphorus, calcium and magnesium bioavailability from lupin (Lupinus albus var. multolupa)-based diets in growing rats: effect of α-galactoside oligosaccharide extraction and phytase supplementation. British journal of nutrition, 2006, 95.06: 1102-1111.

Pryde, S. E., et al. The microbiology of butyrate formation in the human colon. FEMS microbiology letters, 2002, 217.2: 133-139.

Rada, V.; Petr, J. Enumeration of bifidobacteria in animal intestinal samples. VETERINARNI MEDICINA-PRAHA-, 2001, 47.1: 1-4.

Rada, V. Využití probiotik, prebiotik a synbiotik. Interní medicína pro praxi (2010): 92-97.

Rashid, M.; Sultana, M. Role of Probiotics in Human and Animal Health Review. Journal of Probiotics & Health, 2016, 2016.

Reid, G., et al. New scientific paradigms for probiotics and prebiotics. Journal of clinical gastroenterology, 2003, 37.2: 105-118.

Rubio, Luis A., et al. Ileal digestibility of defatted soybean, lupin and chickpea seed meals in cannulated Iberian pigs: II. Fatty acids and carbohydrates. Journal of the Science of Food and Agriculture, 2005, 85.8: 1322-1328.

Ruiz, M. A.; Sotelo, A. Chemical composition, nutritive value, and toxicology evaluation of Mexican wild lupins. Journal of Agricultural and Food Chemistry, 2001, 49.11: 5336-5339.

Salmanowicz, B. P. Capillary electrophoresis of seed 2S albumins from Lupinus species. Journal of Chromatography A, 2000, 894.1: 297-310.

Scardovi, V.; Zani, G. Bifidobacterium magnum sp. nov., a large, acidophilic bifidobacterium isolated from rabbit faeces. International Journal of Systematic and Evolutionary Microbiology, 1974, 24.1: 29-34.

Scardovi, V.: Genus Bifidobacterium. Mair NS (ed) Bergey's Manual of Systematic Bacteriology. 1989.:1418 – 1434

Scholz-Ahrens, K. E., et al. Effects of prebiotics on mineral metabolism. The American journal of clinical nutrition, 2001, 73.2: 459s-464s.

Smith, S. C., et al. Lupin kernel fiber consumption modifies fecal microbiota in healthy men as determined by rRNA gene fluorescent in situ hybridization. European journal of nutrition, 2006, 45.6: 335-341.

Sonnenborn, U.; Schultze, J. The non-pathogenic Escherichia coli strain Nissle 1917–features of a versatile probiotic. Microbial Ecology in Health and Disease, 2009, 21.3-4: 122-158.

Stibůrek, O.; Příbramská, V.; Lata, J. Místo probiotik (nejen) v léčbě gastrointestinálních chorob. Interní Medicína pro praxi, 2009, 11 (1): 25 – 29.

Suchý, P., Straková, E. 2015. Lupiny jako zdroj proteinu. Zemědělec, 32, 15.

Švestka, T. Mikroflóra trávícího traktu a probiotika. Pediatria pre prax, 2007, 1: 34-35.

Tannock, G. W. Commentary: remembrance of microbes past. International journal of epidemiology, 2005, 34.1: 13-15.

Thompson, C. L.; Wang, B.; Holmes, A. J. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME Journal, 2008, 2: 739-748.

Tuohy, K. M., et al. Modulation of the human gut microflora towards improved health using prebiotics-assessment of efficacy. Current pharmaceutical design, 2005, 11.1: 75-90.

Van Barneveld, R. J. Understanding the nutritional chemistry of lupin (Lupinus spp.) seed to improve livestock production efficiency. Nutrition Research Reviews, 1999, 12.02: 203-230.

Vlková, E.; Rada, V.; Trojanová, I. Enumeration, isolation and identification of bifidobacteria from dairy products. Acta agriculturae slovenica, 2004, 84: 31-36.

Vlková, E.; Bunešová, V.; Rada, V. Podávání bifidobakterií mláďatům přežvýkavců. Veterinářství, 2013, 63 (5): 371 – 374

Volek, Z., Skřivanová, V., Marounek, M, 2004, Obsah vlákniny ve výživě králíků, Zemědělec, 48, 10.

Volek, Z.; Marounek, M. Whole white lupin (Lupinus albus cv. Amiga) seeds as a source of protein for growing-fattening rabbits. Animal feed science and technology, 2009, 152.3: 322-329.

Volek, Z., Marounek, M. Effect of feeding growing–fattening rabbits a diet supplemented with whole white lupin (Lupinus albus cv. Amiga) seeds on fatty acid composition and indexes related to human health in hind leg meat and perirenal fat. Meat science, 2011, 87.1: 40-45.

Volek, Z., et al. Effect of diets containing whole white lupin seeds on rabbit doe milk yield and milk fatty acid composition as well as the growth and health of their litters. Journal of animal science, 2014, 92.5: 2041-2049.

Von Buneau, R., et al. Escherichia coli strain Nissle 1917: significant reduction of neonatal calf diarrhea. Journal of dairy science, 2005, 88.1: 317-323.

Wang, S., Errington, S., Yap, H. H. Studies on carotenoids from lupin seeds. In: Lupins for Health and Wealth'Proceedings of the 12th International Lupin Conference. 2008. p. 14-18.

Wu, H. – J.; Wu, E. The role of gut microbiota in immune homeostasis and autoimmunity. Gut microbes, 2012, 3.1: 4-14.

Zdunczyk, Z., et al. The composition and enzymatic activity of gut microbiota in laying hens fed diets supplemented with blue lupine seeds. Animal Feed Science and Technology, 2014, 191: 57-66.

Zdunczyk, Z., et al. Determinants and effects of postileal fermentation in broilers and turkeys part 2: cereal fibre and SBM substitutes. World's Poultry Science Journal, 2015, 71.01: 49-58.