University of Veterinary Medicine

Department of Pharmacology and Toxicology

Gene expression status of beta-lactam resistance in certain probiotic bacteria

by

Kristin Skarsboe

Supervisor:

Dr. Orsolya Palócz
Research fellow

Hungary, Budapest

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1. Introduction

One of the major crises we are facing today is antibiotic resistance. The antimicrobial usage of both humans and animals has intensified within the last 50 years. Now, several types of antibiotics are no longer effective, making it harder to treat bacterial infections. It is therefore important to reduce antibiotic usage to more severe cases and find new types of medicine that can replace them, such as probiotics [1].

With the increased administration of antibiotics, new laws and restrictions follows. Not only have the regulations become stricter in Europe, but WHO, World Health Organization also works on optimizing the usage of antimicrobials [1].

Probiotics have had an increased interest due to their proven beneficial effect of both gastrointestinal tract and reproductive system [1], this paper will focus on the vaginal microflora of dairy cows.

The normal vaginal microbiome serves as a defence system against infections of the reproductive system. Reproductive tract infections in cows are one of the key factors that negatively impact the dairy industry, resulting in a significant yearly economic loss. It is also likely one of the major factors that affect and contribute to infertility in postpartum dairy cows [2].

The frequent and sporadic use of antibiotics to treat diseases of the reproductive system in cows has been connected to growing indications of microbial resistance and antibiotic residues in milk. Therefore, it is critical to consider alternative therapies for cows with illnesses of the reproductive tract [2].

The most prescribed, and most effective antibiotics in use today are the beta-lactams. However, there are already numerous bacteria that have created resistance against these types of antibiotics, such as MRSA, methicillin-resistant-*Staphylococcus-aure*us and VRE, vancomycin-resistant *Enterococci [3].* Two common disorders of dairy cows treated with antibiotics are mastitis, also termed; mammary gland inflammation, and acute puerperal metritis [4].

Mastitis is one of the most critical diseases in the dairy industry because of its high prevalence and huge economic impact. At any given time, up to 50% of all dairy cows have mastitis in some form. About 70% of antibiotics used in dairy farming are for udder health, primarily for treatment of non-lactating cows [4].

Within 21 days of parturition, acute puerperal metritis, a systemic disease, affects the dairy herd's ability to reproduce and generate income. The only possible treatment yet for this

is also antibiotics. Administration can vary from intrauterine to systemic, in some cases both are applied subsequently. However, there is a significant failure rate between 23% and 35% when evaluating results following antibiotic treatment. When adding up the numbers with the increase of antibiotic-resistant genes, it is obvious that measurements are needed to be implemented to reduce the occurrence of these genes and to maintain a healthy environment for the food-producing species [4].

Probiotics are one of the best options today to encourage a healthy microbial balance. and to ensure a responsive immune system to prevent increasing antibiotic resistance while still having effective antibiotics available. Research on probiotics in the food sector is crucial due to the yearly increase in demand for food production [5].

Probiotics are already widely used, particularly for food-producing animals like fish, ruminants, and poultry. *Lactobacillus, Bifidobacterium, Lactococcus, Bacillus, Streptococcus*, and certain yeasts are the most often utilized types [5].

However, with the increasing usage of probiotics, the world is now facing new issues requiring further research. Probiotics, most being bacteria themselves, often applied together with antibiotics, seem to have developed antibiotic-resistant genes. Probiotics, being non-pathogenic and wanted bacteria for both the internal environment and immune system of the host, resistance could possibly be a positive side effect. However, since bacteria can transfer genes between themselves, no matter the type, they have the potential to transmit these genes to the pathogenic bacteria. If this is the case, antibiotic resistance will only spread faster, with devastating results [6].

The concerns outlined above are just the tip of the iceberg of why further research as well as reviewing existing studies and screening of probiotics are needed for us to safely administer probiotics [6].

2. Literature review

2.1. Normal microbiota in the vagina of cows

The protection against infections of the reproductive system is provided by the natural vaginal microbiota. One of the main elements that have a detrimental economic effect on the dairy business is the reproductive tract infection in cows. Additionally, it is probably one of the main elements that influences and contributes to infertility in postpartum dairy cows. The annual economic losses caused by uterine infections in cows are massive [7].

The normal microbiota of the cows' vagina is different between individuals and depends on their health status. The optimal pH of the vagina of cows lies at 7.3, plus minus 0.63 [8]. The existing microbiota varies in types of bacteria from aerobic to anaerobic, the most dominant ones being Gram-positives. Aerobic such as *Staphylococcus, Streptococcus,* and coliforms are found together with facultative anaerobic such as *Lactobacillus, Fusobacterium,* and *Peptostreptococcus [9]*.

The information of physiological vaginal flora in cows are limited, and extended research has not yet been performed. Most of the research is based on uterine cultures, making it difficult to collect the needed data [10].

2.2. Endometrial diseases

In case of any abnormalities, pathogenic bacteria can delay uterine involution, induce endometrial lesions, cause inflammation, and interfere with embryo survival. Additionally, postpartum ovarian follicular growth and function are disturbed because of uterine bacterial infection, bacterial products, or the accompanying inflammation that prevents cows from ovulating. As a result, uterine illness is linked to lower conception rates, longer intervals between calving and the first service or conception, as well as a higher incidence of cattle that are culled due to infertility [11].

Previous research has tested vaginal probiotics for the prevention and treatment of bovine reproductive system infections. Comparing the vaginal microbiology of healthy cows and those with endometritis may indicate probiotics for the prevention and treatment of endometritis. More research is needed, however, studies on humans, unrelated to bovine, have revealed that the dominant strains of vaginal microbiota can be used to prevent and treat vaginal infections by acting as a biological barrier or by producing lactic acid, bacteriocins, and hydrogen peroxide [7].

Both benign and malignant uterine illnesses are correlated with endometrial microbiome dysbiosis. An effective substitute for hormone and antibiotic therapy in the treatment of uterine illness in cattle is the intravaginal infusion of symbiotic bacteria. Nutrition can also be used to mimic genital microbial diversity since an energy-balanced diet promotes the formation of microbial populations. It is possible that probiotics that change the endometrial microbiota could offer effective substitutes for the current treatments for uterine illness [12].

Acute puerperal metritis, APM, is an infection of the uterus that develops within 21 days of parturition and is distinguished by the uterus being enlarged with discharge. The discharge characteristics vary from red-brown watery to viscous whitish purulent that frequently has a foul smell. It is a systemic disease, acute phase with a fever of 39.5 °C or above, together with symptoms of toxaemia. The disease is widely recognized. Indicators of APM such as reduced milk production, dullness and/or other toxaemia-related symptoms, lower dry matter intake, higher heart rate, as well as dehydration are often seen. APM is one of the most significant postpartum disorders in dairy cows due to its grave negative effects on both economics and reproductive function [13].

Numerous bacteria, primarily *Escherichia coli, Fusobacterium necrophorum,* and *Trueperella pyogenes,* are the major causes of metritis. Antibiotics are frequently used to control the condition due to its considerable economic loss accompanied by milk withdrawal, and impaired infertility. The drug choice for treating metritis is the third generation cephalosporins. However, due to antibiotic resistance, these antibiotics are more and more limited to human medicine. There is already evidence that treatment with probiotics intravaginally can have better outcomes than with only antibiotics [12].

Mastitis is an infection of the mammary glands. The indication of an infection consists of several factors, such as genetics, feed, supplements, environment, infection risk, milking techniques and overall hygiene. It is usually caused by microbes entering from a dirty environment, by injury, or systemically. If the cow has a decreased immune system, such as during calving or in situations increasing stress, less pathogens is needed to form an infection. Dairy cows are affected in higher percentage compared to beef cows, occurring more commonly not long after calving [14].

2.2.1. Antimicrobial usage

Growing evidence of microbial resistance and antibiotic residues in milk have been linked to the routine and sporadic use of antibiotics to treat cows' reproductive system infections.

Therefore, it is crucial to assess alternate treatments for cows with reproductive tract diseases [7].

When antibiotics first came to the market, there were no restrictions nor rules on how and when to administer them. This resulted in misuse, causing the bacteria to become resistant. It is not until the 21st-century, regulations have been made. One of the most recent regulations entered into force on 28th January 2022, applying to all countries of the European Union [1]:

- *A ban on the preventative use of antimicrobials, both in groups of animals and in medicated feed.*
- *Restrictions on metaphylactic use of antimicrobials.*
- *A reinforced ban on the use of antimicrobials to promote growth and increase yield.*
- *The possibility to reserve certain antimicrobials for humans only.*
- *The obligation for Member States to collect data on the sale and use of antimicrobials.*
- *For imported animals and products from outside the EU, a ban on antimicrobials for growth promotion and restriction on antimicrobials reserved for human use.*

[1, 15]

2.3. Beta-lactams

The beta-lactam antibiotics are still the most popular used antibiotics today. Methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, *Enterobacteriaceae*, and *Acinetobacter baumannii* are the four primary bacterial species that cause most antibiotic-resistant infections in medicinal context [3]. Also, *Staphylococcus pneumoniae* and *Pseudomonas aeruginosa* are a large threat globally to both animal and human health [16].

The beta-lactam antibiotics share the highly reactive 3-carbon and 1-nitrogen ring, termed the beta-lactam ring, as a biological characteristic. They are divided into penicillin, cephalosporins, carbapenems, monobactams, and beta-lactamase inhibitors. Due to resistance, combinations, and modification of these are also more frequently used [16].

From February 9th, 2023, all usage of carbapenems and monobactams became prohibited for veterinary medicinal administration, making them reserved for specific antimicrobial treatments for humans [17].

A crucial component of the bacterial cell wall that gives its mechanical rigidity is peptidoglycan, often known as murein. Both the Gram-positive and Gram-negative cell walls contain this highly conserved component. Even so, peptidoglycan is a dense structure in Gram-positive bacteria, approximately 10 layers, whereas in Gram-negative it only consists of one or

two layers. The composition of peptidoglycan is constituted of glycan chains built by subunits of N-acetylglucosamine and N-acetylmuramic acid disaccharide [16].

In case of treatment of metritis, antibiotics are administered intrauterine, systemically, or both. For systemic administration, antibiotics including penicillin, third generation cephalosporins, and ampicillin are commonly used. Additionally, combinations of antibiotics such as systemic penicillin or ampicillin along with cloxacillin with either oxytetracycline or ampicillin intrauterine have been popular to administer against APM in dairy cows [13].

Clinical mastitis (CM) in dairy cows are firstly managed locally by intramammary administration, in case of severe mastitis, extra antibiotics can be given parentally as well. Also, as an additional treatment to reduce the incidence of mastitis, on the day of drying-off, a local injection of antibiotic is given, roughly six weeks prior to the subsequent calving. This treatment of all cows during their dry-off period has resulted in less occurrence of CM, accompanying a reduction of mastitis-causing pathogens and eradication of certain pathogens from the herd. This treatment is termed the dry cow treatment, DCT [18].

However, recent studies have shown that zoonotic germs are becoming more resistant to antibiotics in food animals around the world, raising concerns for public health. Growing antibiotic resistance is linked to declining clinical effectiveness and ramifications on animal welfare as well as the economy [13].

2.3.1. Cephalosporins

We distinguish five generations of cephalosporins. Most Gram-positive bacteria are already resistant to the active agents in the first generation, while higher generations often have a broader spectrum for Gram-negative aerobic bacteria. MRSA, a Gram-positive bacterium is only susceptible to the fifth generation of cephalosporin, being ceftaroline and ceftobiprole [19].

There are several different types of third generation agents. The fundamental beta-lactam structure has been chemically altered to produce all the third generation cephalosporins that are currently on the market. Cephalosporins, like other beta-lactam medicine, prevent the cross-linking of peptidoglycans and binds to and deactivate PBPs, penicillin-binding proteins, which prevents the formation of the bacterial cell wall [20].

Cefotaxime, ceftizoxime, and ceftriaxone are third generation medicines with an aminothiazolyl substitution at the R1 position that enhances affinity to PBPs and boost efficacy against Gram-negative bacteria. Increased resistance to bacterial beta-lactamase is the result of substitution at the R1 site, any alteration at the R2 site can impact toxicity and lengthen half-life [20].

2.4. Antimicrobial resistance

There are various ways that resistance genes are acquired. Gene mutation or the acquisition of external resistance are both methods of achieving resistance. Large transferable plasmids that carry a range of resistance genes are also possible. Cointegrates can be formed between plasmids and transposons that contain one or more resistance genes. While some plasmids can be activated by a coresident transferable plasmid, others are encoded with their own transfer machinery. Additionally, chromosomal components can move independently or be activated by plasmids that can again be transferred [21].

Beta-lactamases are the primary method by which bacterial resistance to beta-lactams manifests themselves, although there are additional processes as well. These mechanisms vary between synthesis of beta-lactamases for inactivation, reduced penetration to the target site, modification of PBPs target site, and efflux through certain pumping mechanisms from the periplasmic region [16].

Antibiotics can freely enter the cytoplasmic membrane of Gram-positive bacteria, where the PBPs are found. However, in Gram-negatives; the bacterial outer membrane, which is lacking in Gram-positives, can both limit beta-lactam entrance as well as concentrate beta-lactamase molecules. Even a very weak beta-lactamase can bestow large levels of resistance if beta-lactam molecules are significantly restricted from the periplasmic region. This done so by either reducing entry or increasing efflux, as well as if the beta-lactamase molecules are highly concentrated [21].

Enzymes produced by bacteria termed beta-lactamases hydrolyses the beta-lactam ring of the beta-lactam antibiotics, resulting in antibiotic resistance. These enzymes are encoded by several different genes carried by motile genetic elements, most often being plasmids. The beta lactamases are divided further into classes; Class A, B, C, and D [6, 22, 23].

Class A beta-lactamases hydrolyses penicillin and the older generation of cephalosporins, however, the subgroup extended-spectrum beta-lactamases (ESBLs), hydrolyses the third generation as well. These enzymes can be found in *Escherichia coli* and *Klebsiella spp.*, along with others. Class D, also termed OXA-type, hydrolyses penicillin, especially oxacillin. They are more commonly found in *Acinetobacter spp.* and members of the order *Enterobacterales* [6, 22, 23]. The beta-lactamases genes are DNA segments encoding the production of these enzymes [23].

A single *FtsH* gene is carried by most bacteria, which are membrane-bound, ATP dependent, zinc metalloproteases. It does numerous important processes within the bacteria to

remain its stability, degrading abnormal proteins, cell membrane integrity regulation and modulates heat stress response. By having the ability to alter the before mentioned processes, it can as well alter the drug efflux and transportation by maintaining the membrane proteins, possibly creating antibiotic resistance [24, 25].

The Class A beta-lactamase gene *PenP* is found commonly in the Bacillus species. They protect the bacteria by neutralising the beta-lactam antibiotics due to their serine hydrolases, contributing to antibiotic resistance [23, 26].

RepN works during bacterial replication, it is a replication protein that makes sure the genes in the plasmid, that might carry antibiotic resistance, are transferred to the next bacteria [27, 28].

The ypxI is a class D beta-lactamase produced by *Bacillus subtilis*. Class D enzymes of Gram-positive bacteria have a different structure and use unique substrate binding that is vastly different from the other classes of beta-lactamases [29].

To illustrate the seriousness of antimicrobial resistance, we can look at MRSA as an example which started mainly as a serious issue in swine, and was spreading to other food-producing animals, such as dairy cows. Clinical illness is yet uncommon and most animals with MRSA are asymptomatic carriers, however, there have been instances of MRSA in dairy cow milk and occasionally in connection with mastitis. The largest antibiotic groups such as penicillin, tetracycline, cephalosporins, and possibly other antibiotics have all been found to be ineffective against MRSA in cattle. This suggests that there may be few to no antibiotics that will be effective in case the condition of MRSA evolves to become a prevalent cause of mastitis [30].

2.5. Probiotics

The meaning of the word probiotics is ‘for life’ and originates from Greek. It has had several different definitions throughout the 20th century, resulting in 1989 with ‘*A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’*, stated by Fuller, excluding the viable probiotics from the previous definition. The use of probiotics can be tracked all the way back to the Sumerians in year 2500 B.C. with fermented milk by inoculation [31].

Ilja Metchnikoff was one of the first scientists researching probiotics, specifically the benefits of yogurt in the gut microflora. For the studies, he used the then-called ‘Bulgarian bacillus’, today, known as *Lactobacillus delbrueckii subsp. bulgaricus*. This subspecies is still used in yogurt production. The research of probiotics declined during the First World War and

after his death in 1916. However, post-war during the 1920s, this bacterium was replaced with *L. acidophilus* which had promising results. Then Second World War came and again put a pause on probiotic research. After the war, it was revived due to the invention of antibiotics and increased interest in germ-free animals. It was then discovered that the research of *L. acidophilus* as the dominant bacterium was incorrect and so the *Bifidobacterium* took over its place [31].

The definition used today for probiotics comes from FAO/WHO in 2002, this definition says when administered correctly, the active microbes have a positive effect on the health of the animal [5].

Probiotics are not necessarily specific and can range from microorganisms to bacteria, to yeast. Their most important site of function is in the gastrointestinal tract, here they work against- and neutralize pathogenic microorganisms [32]. They are administered orally and are usually recognized as safe products, however in some cases, it can impede with the physiological microbiota causing an opportunistic growth of bacteria or fungi. Considering the amount of probiotics that are daily consumed by both humans and animals, it is important to have a safety assurance of the products [33].

Application of probiotics is mainly by oral administration, there is limited research done on intra-vaginal and intra-uterine application. There is however, research done for intra-uterine application by infusion of *L. buchneri* in cows resulting in lower occurrence of infective diseases of the uterus as well as higher percentage of pregnancies. Additionally, it reduced the mucosal adhesion of E. coli by inhibiting lipopolysaccharide secretion. An *in vivo* study mixing *Lactobacillus* and *Pediococcus* administered with an intra-vaginal pessaries was also found to reduce the incidences of metritis [34].

To avoid further antimicrobial resistance and still have effective antibiotics, probiotics are one of the best options to promote a good microbial balance and to ensure a responsive immune system. The demand for food production is increasing rapidly each year, which is why the research on probiotics in the food industry is so important. [5]. Already, the usage of probiotics is increasing rapidly, especially for food-producing animals such as poultry, ruminants, and fish. The most common ones used are *Lactobacillus, Bifidobacterium, Lactococcus, Bacillus, Streptococcus,* and some yeasts [5].

2.5.1. *Bacillus* genus

The *Bacillus* probiotics are used for food-producing animals, as well as humans. These bacteria have been a popular choice for 50 years, at least. The most common ones are *B. subtilis,*

B. clausii, B. cereus, B. coagulans and *B. licheniformis*. The *Bacillus* species are heat-stable giving them several advantages such as room temperature storage and low pH resilience. The last mentioned advantage is making the entire amount administered effective in the small intestine, compared to *Lactobacillus* which are not pH resilient, resulting in some of the substance being dissolved by the gastric juice [35].

These spore-forming probiotics, such as the *Bacillus spp*., are used for several reasons within food-producing animals, such as growth promoters, competitive exclusion agents as well as in aquaculture [35].

Bacillus subtilis is a transitory digestive tract bacterium with the ability to produce spores that withstands both heat and cold. The microbe is said to be able to improve immunity and diet digestibility as an animal-feed probiotic. It can also boost anaerobiosis, encouraging the growth of native lactobacilli which again produces lactic acid and limits the growth of harmful bacteria [36]. *B. subtilis* produces specific bacteriocins such as subtilin, sublancin, bacillocin and subtilosin, while *B. licheniformis* produces bacitracin and lichenin. These bacteriocins of the *Bacillus* bacterium result in a broad spectrum of inhibitory activity [37].

Both *Bacillus subtilis* and *B. licheniformis* have a very high branched-chain fatty acid, BCFA, concentrations in their cell walls. Adding different types of *Bacillus* to the dairy cow diet, it is linked with several beneficial effects, such as enhancing fibre digestibility, increasing milk yield and its composition, as well as lowering enteric methane emissions [38].

One of the OIE/IABS international conference "Alternatives to antibiotics" provided proof that a correct mixture of natural antibacterial peptides, biological response modifiers, pre-, and probiotics, along with proper development of the gut microbiome, can reduce the use of antibiotics in food-producing animals. In disease models of cows, some of the aforementioned methods have been effective. There is evidence that probiotics can make a significant contribution to illness prevention in dairy cattle herds [39].

The conferences' findings include expanded farm interventions, such as:

- The early identification of disease symptoms, enabling quicker and more efficient medication therapies.
- A cost-benefit study of recurrent antibiotic treatments for the convenience of farmers and the health of the animals.
- An updated diagnostic method for production diseases. Here, there is a need for clinical immunology and chemistry tests to forecast production disorders on reliable, approachable criteria linked to subpar environmental adaption and pertinently high disease occurrence risk in cattle [39].

Probiotics in cattle have been used to treat diseases such as subacute ruminal acidosis (SARA), metritis, Johne's disease, and diarrhoea in calves. Probiotics have also been successfully employed in cattle to treat the clinical symptoms of heat stress and its corresponding interruptions to milk supply and reproductive tract functioning. Additionally, they improve both disease and health condition. Immunomodulatory effects can also be seen by increasing ruminal microbial fermentation, feed digestibility, and -conversion efficiency. As a result of these impacts, dairy cows may produce more milk and have better milk quality, while beef cattle may perform better in terms of growth. However, these positive outcomes are not guaranteed, and several show no signs of any effect [40].

2.5.2. Probiotics resistance

The risk of probiotics transferring their genes, possibly resistance genes, increases day by day. Probiotics are often administered simultaneously with antibiotics, creating an ideal environment for the probiotics to develop resistance genes. Transfer of plasmids, transposons, and genetic information has already been reported. The most common transferring method is horizontal, divided further into conjugation, transduction and transformation [6].

Conjugation is often carried out by plasmids, by a direct connection of the cells. Plasmid DNA is then transferred by the means of a pilus connected from a donor bacterium to the recipient bacterium [23].

Transduction is performed by bacteriophages, a virus that has infected the bacterial cell. The phages replicate within the bacteria and package its DNA. They then continue to infect other bacteria, bringing along the DNA [23].

Transformation requires no contact between the bacteria, the DNA is freely in the environment, released by lysed cells. The probiotics integrates these extracellular DNA, found more often in the gastrointestinal tract [23].

The factors influencing the genetic transfer varies greatly. The resistance genes located on the mobile genetic elements such as plasmids and transposons have a definite higher risk of spreading by horizontal transfer. The environmental conditions also have a key role in providing an ideal environment for gene transfer, by particularly creating a selective pressure. During antibiotic exposure there is an obvious encouragement to achieve resistance genes [24, 26].

Lastly, the microbiota influences the behaviour, adaptation and interaction between the bacteria. Previously mentioned, there is lack of research of the vaginal microbiota of dairy cows, so the interactions here are not yet fully understood. Considering the gut microflora, with

the high bacterial density, there is a large interaction between probiotics and the pathogenic bacteria [6].

Although the majority of bacteria are benign and probiotics are considered safe substances, there are several circumstances when a host's health may deteriorate to various degrees as a result of the environment between the host and the bacteria. When the host is stressed, due to the defence system or by external stress, the virulence factors may increase as well due to genomic changes [41].

2.6. One Health

'One Health' is a strategy aimed at maintaining the health of people, animals, and the natural environment through cooperative problem-solving at local, national, and international levels. The concept concentrates on zoonotic diseases that may result in endemics or pandemics and focuses especially on pathogens where antibiotic resistance can arise, spreading between humans, animals, and environments. It is unlikely that effective mitigation methods will be developed if today's health issues are solely approached from one side only. No matter if it's medicinal, veterinary, or ecological point of view since the issues are usually complex, zoonotic, widespread, and with several factors to consider [42].

To underline the seriousness of current situation we are in today, and its consequences: infections with multi-resistant bacteria result in the deaths of about 700,000 people each year.

According to the report the UK government ordered in 2014, 10 million casualties will take place by diseases brought on by bacteria with antimicrobial resistance in 2050 if a reduction of resistance is not observed [18].

Including patient and hospital expenditures, the overall crude economic burden of antibiotic resistance was estimated to be at least 1.5 billion euros in 2017 in Europe, and 55 billion dollars in the USA in 2000. Based on lost wages brought on by illness or early death, indirect patient costs were calculated. Productivity losses made up about 40% of the total projected 1.5 billion euros in the European estimates, compared to 64% of the American estimates [43].

3. Goals

The aim of this project was to provide scientific data on how the different antimicrobial drugs affect the antimicrobial resistance of probiotics. We applied bacteria that were isolated from healthy dairy cows' vaginal microbiota. These probiotics candidates are our supposedly beneficial bacteria for use in the outer genital tract of cows as probiotics for prevention of metritis. We would like to measure the expression of the beta-lactam resistance genes after antimicrobial treatment in these probiotic candidate bacteria *in vitro.* With these tests, we would like to obtain data on the safety of these probiotics and whether they can be safely administered to farm animals.

The bacteria used:

- *Bacillus licheniformis*
- *Bacillus pumilus*
- *Bacillus subtilis*

The antibiotics used:

- Amoxicillin
- Cefquinome
- Ceftiofur

4. Materials and methods

4.1. Microplate broth dilution

The CLSI (2015) certified broth microdilution technique was applied. 96-well sterile microplates with flat bottom were employed for the assays. *Bacillus licheniformis, Bacillus pumilus* and *Bacillus subtilis* were examined. These bacteria were isolated from the vaginal microbiota of healthy dairy cows.

Using a multichannel micropipette first, 100 µl tryptic soy broth (TSB) containing the antimicrobial substances in a 10-fold serial dilution (500 mg/l to 0.05 mg/l) was added to each well of the microplate. The tested antimicrobial substances were: amoxicillin trihydrate, ceftiofur hydrochloride and cefquinome sulphate. To the control wells (0 mg/l) 100 µl TSB was added. Secondly, the inoculation of bacteria was made in a final concentration of 10^4 CFU/ml. The dishes were cultivated for 24 hours at 37 °C. After that, the absorbance of each plate was measured at 600 nm in a microplate reader to detect any level of bacterial growth.

4.2. Treatment of bacteria

According to the MIC results amoxicillin trihydrate and cefquinome sulphate were chosen for further analysis. The antimicrobial substances were used in sub-MIC concentration, the amoxicillin in 0.5 mg/l and the cefquinome sulphate was used in 0.05 mg/l. The test materials were diluted it TSB freshly before the experiment. The bacterial suspensions (*Bacillus licheniformis, Bacillus pumilus* and *Bacillus subtilis*) were applied in 10^4 CFU/ml density and treated in 6-well plates; 4 ml/well for 24 h at 37 °C at 90 rpm. The control bacterial suspensions were received only TSB. The experiments were done in 6 parallels.

After the experiment, the bacterial suspensions from each well were separately measured into the 10 ml centrifuge tubes and were centrifuged at 3000 g for 10 minutes. From the formed pellet that contained the bacteria RNA was isolated.

4.3. RNA isolation

RNA was isolated with Ribopure Bacteria Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The isolated RNA was eluted by adding 25 µl elution solution.

4.4. Reverse transcription and quantitative PCR

The Maxima H Minus First Strand cDNA Synthesis Kit (ThermoScientific) was applied for reverse transcription of 1000 ng mRNA from each sample, during the cDNA synthesis procedure we strictly followed the manufacturer's instructions.

We performed the quantitative PCR analyses on the CFX Opus Real-Time PCR System (BioRad). The tested and the reference genes are given in Table 1. The final reaction volume of 20 µl contained 0.2 µM of the corresponding primers and 1× concentrated SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in nuclease-free water, and the 2 µl cDNA sample which was added directly to a PCR reaction mixture for each PCR reaction. PCR reactions were run at a thermal cycle of 95 °C for 3 minutes, followed by 40 cycles at 95 °C for 20 seconds, then at 60 °C for 30 seconds and then at 72 °C for 30 seconds. At the end of each cycle there was a 10-second-long fluorescence monitoring. After the 40 cycles melting curve analysis was performed.

Table 1. Sequence of primer sets used for quantitative PCR

Species	gene symbol	gene		primer	base pair	
Bacillus licheniformis	*PenP*	beta-lactamase class A	F	GCAATCACTCGAATGCCTCAC	178	test
			R	ATCGTCGATGCAAAAGCGAAG		
	ITS	internal transcribed spacer	F	ATGCCGCGGTGAATACGTTC	161	reference
			R	CACCTTCCGATACGGCTACC		
	FtsH	ATP-dependent zinc metalloprotease	F	CCTGGAACGGGTAAATCGCT	214	test
			R	GGTCCGATGAACTCATGCCT		
Bacillus pumilus	*MFS*	tetracycline resistance MFS efflux pump	F	ATTGTCGGACCGAGCCTTG	141	test
			R	AGAAACTGTCGAAGGATGCTG		
	ITS	internal transcribed spacer	F	TATATGGAGCAGCGTGCGTT	226	reference
			R	CATCGGCTCCTAGTGCCAAG		
	ypxI	beta-lactamase class D	F	GAAGAGAAACACGCCACCCT	124	test
			R	TGCCGGTGCCTTTGATATTTG		
Bacillus subtilis	*PenP*	beta-lactamase class A	F	TCTCACGACTGACAAACGCA	122	test
			R	TTCCGGCTCCGGATTTATCG		
	ybxI	beta-lactamase class D	F	AGTTTTGGCTGCAAAGCTCG	168	test
			R	TTCCGGTTTTCCCGTAGAGC		
	repN	replication initiator protein	F	TTTCCAGTAATGAACGGATAGGTC	248	test
			R	CATAATGCAAACTTCTTTAGGCAAA		
	ITS	internal transcribed spacer	F	ACAGAACGTTCCCTGTCTTGT	124	reference
			R	TCACTACGTGATATCTTGCATTACT		

4.5. Statistical analysis

Relative gene expression levels of the genes of interest and statistical analyses were executed by the CFX Maestro Software 2.3. Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc comparison using Tukey's 'Honest Significant Difference' method. Differences were considered significant if the p-value was < 0.05.

5. Results

5.1. Antimicrobial susceptibility results

The effective inhibitory amoxicillin concentration was above 5 mg/ml for the *B. pumilus* and above 50 mg/ml for the *B. licheniformis* and *B. subtilis* (Figure 1). These concentrations are above the clinically effective dose of amoxicillin which is 5-10 mg/kg body weight for *Bacillus* species. Among the three investigated bacteria *B. pumilus* was sensitive to amoxicillin and the *B. licheniformis* and *B. subtilis* were not.

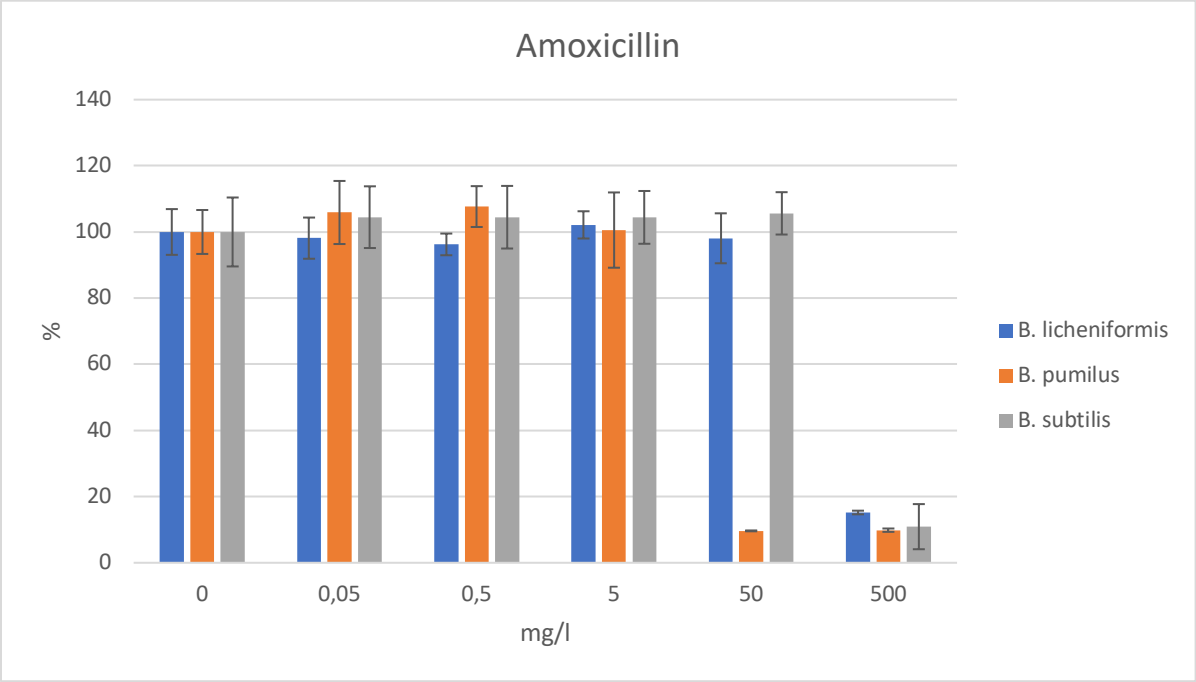

Figure 1. Susceptibility of the tested bacteria to amoxicillin

In figure 1 we can see that the growth of *B. licheniformis* and *B. subtilis* is not inhibited even when 50 mg/l of amoxicillin is administered, while the growth of *B. pumilus* has significantly decreased. These results presume high level of resistance in both *B. licheniformis* and *B. subtilis.* In our experiments their growth was blocked only by the 500 mg/l amoxicillin concentration.

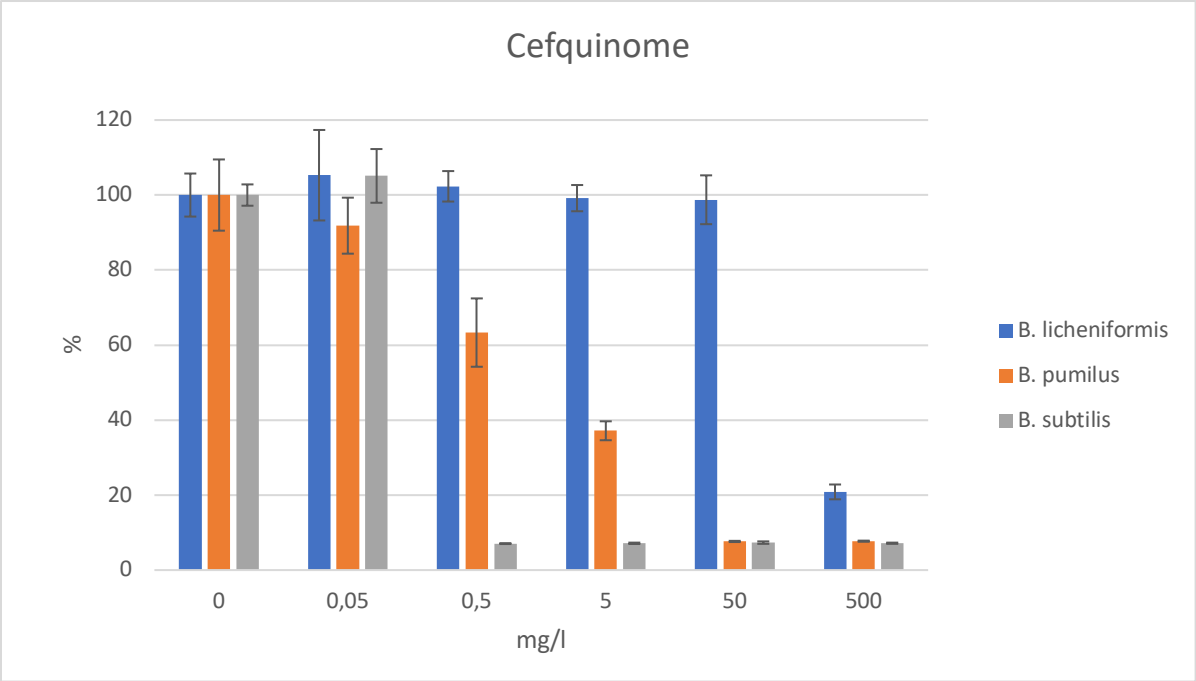

Figure 2. Susceptibility of the tested bacteria to cefquinome

Figure 2 shows how the growth of both *B. pumilus* and *B. subtilis* decreased even at 0.5 mg/l cefquinome concentration, and *B. pumilus* being less sensitive of the two. The growth of *B. licheniformis* however was not blocked until 500 mg/l cefquinome was administered. This surmises the resistance of *B. licheniformis* to cefquinome.

The clinically effective dose of cefquinome against the *Bacillus* genus is 1 mg/kg body weight, hence we can assume that the *B. subtilis* is sensitive, the *B. pumilus* is moderately susceptible, and *B. licheniformis* is resistant to it.

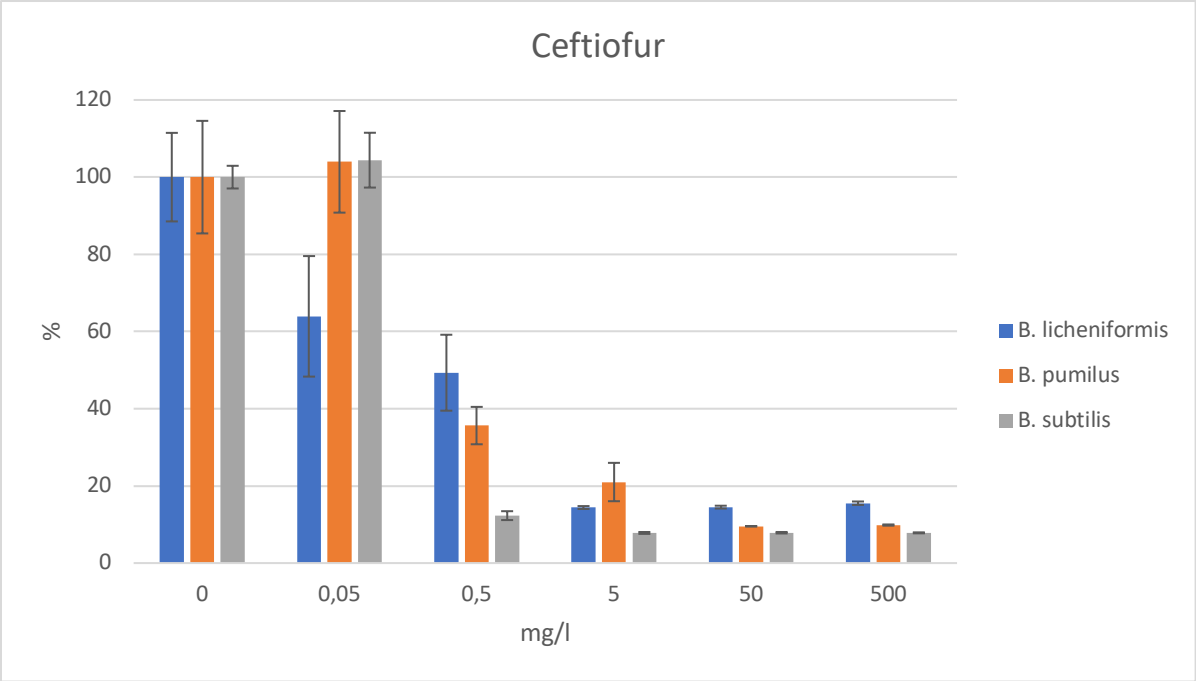

Figure 3. Susceptibility of the tested bacteria to ceftiofur

Figure 3 is showing how the growth of *B. licheniformis* is decreasing at the 0.05 mg/l ceftiofur already, and the growth of *B. pumilus* and *B. subtilis* is decreasing at 0.5 mg/l, therefore all of them sensitive to ceftiofur, since the clinically effective dose of ceftiofur for *Bacillus* species is 1 mg/kg body weight.

5.2. Gene expression results

The gene expression of selected resistance genes was monitored after treatment with 0.5 mg/l amoxicillin or 0.05 mg/l cefquinome. These active substances and their concentrations were chosen after the susceptibility tests. These were the concentrations that had no effect on the growth of the bacilli making them suitable for gene expression measurements. Since these concentrations had no inhibitory effect on the bacilli, we presumed, that some of the resistance genes in the bacteria had already been activated.

The genome of *B. licheniformis* contains an ATP-dependent zinc metalloprotease gene (*FtsH*) which could be responsible for aminoglycoside resistance, it also contains a class A beta-lactamase resistance gene (*PenP*). The expression of the *FtsH* gene was not altered after the treatment with beta-lactam antibiotics (Figure 4). Although the expression of *PenP* gene was increased after both amoxicillin and cefquinome treatment (Figure 4).

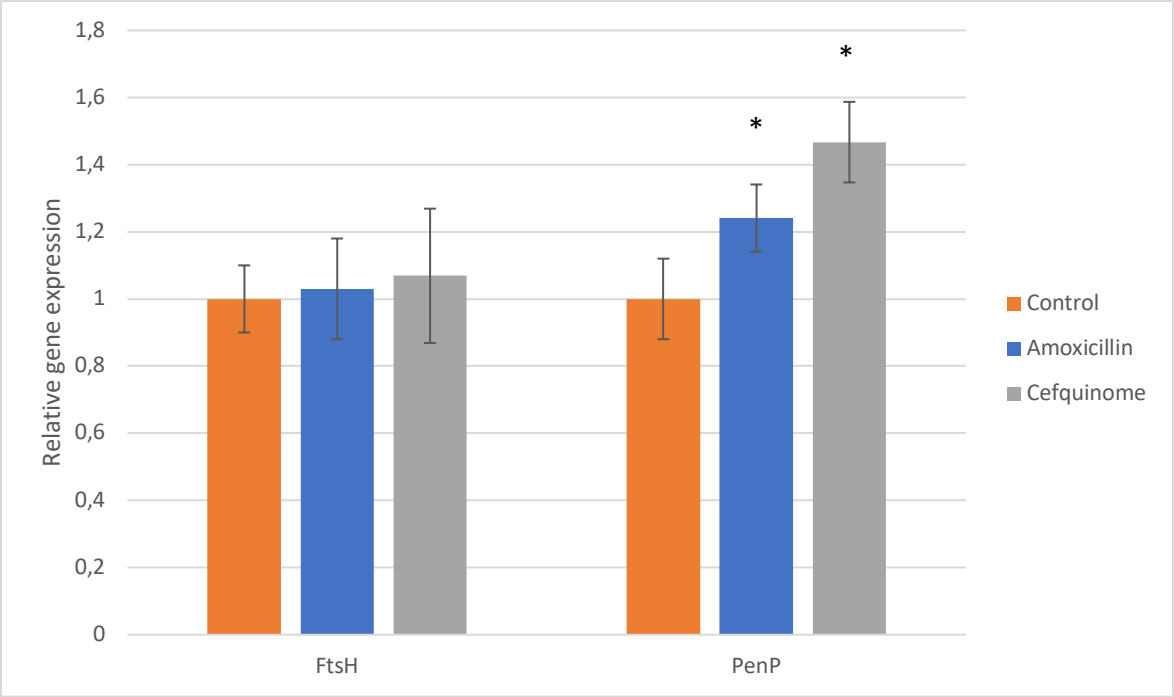

*Figure 4. Gene expression of resistance genes in B. licheniformis after antibiotic treatment, Data are shown as mean±SD. *p<0.05, FtsH – ATP-dependent zinc metalloproteases coding gene, PenP – class A beta-lactamase resistance gene*

The genome of *B. pumilus* contains a tetracycline resistance MFS efflux pump gene (*MFS*) and a class A beta-lactamase resistance gene (*PenP*). The treatment of *B. pumilus* with the amoxicillin (0.5 mg/l) and cefquinome (0.05 mg/l) for 24 hours did not change the gene expression of either *MFS* or *PenP*.

The genome of *B. subtilis* contains the resistance genes *PenP* which is a class A beta-lactamase, and *ypxI* which is a class D beta-lactamase coding gene. After the treatment with amoxicillin both genes were upregulated (Figure 5). However, after the treatment with cefquinome only the *PenP* gene expression elevated (Figure 5).

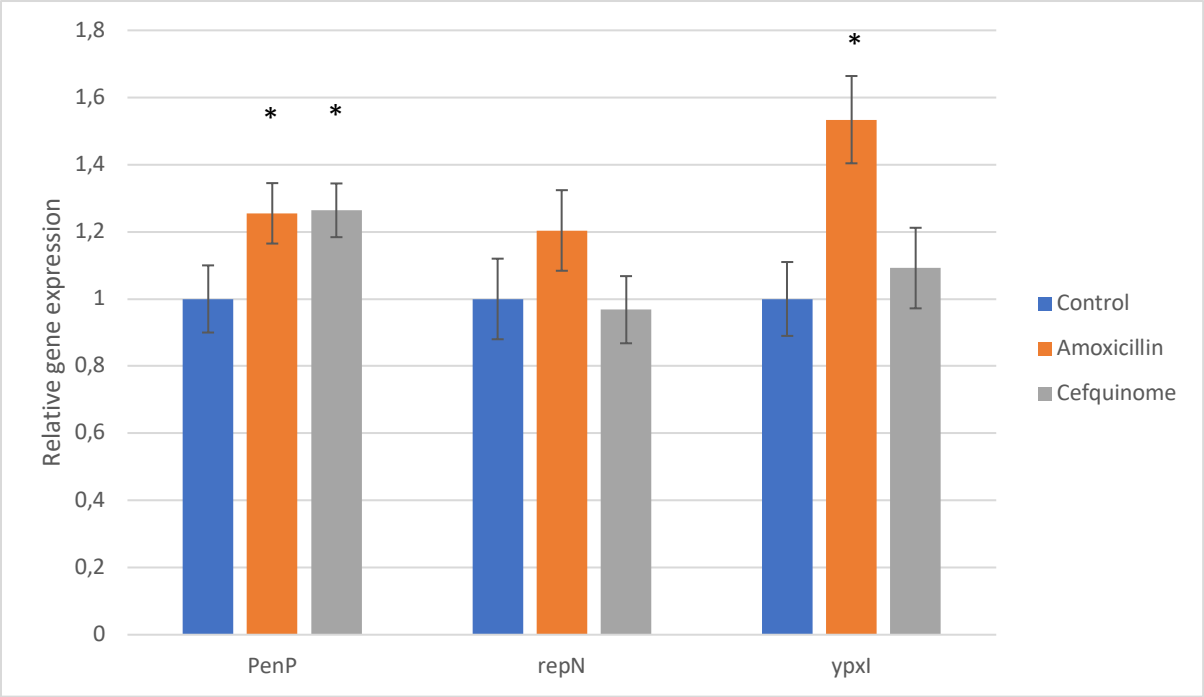

*Figure 5: Gene expression of resistance genes in B. subtilis after antibiotic treatment, Data are shown as mean±SD. *p<0.05, repN – replication initiator protein coding gene, PenP – class A beta-lactamase resistance gene, ypxI - class D beta-lactamase coding gene*

Bacillus subtilis has a plasmid, which possibly encodes several resistance genes. The expression of the plasmid was monitored using the *repN* replication initiator protein coding gene. The mRNA level of *repN* remained unchanged after the treatments with the beta-lactam antibiotics (Figure 5).

6. Discussion

The increase in administration of probiotics in both human and animal health raises questions about their long-term safety, particularly regarding antibiotic resistance. Probiotic usage has increased significantly as common supplements of the daily diet of both humans and animals, as the idea of taking control on one's health has gained popularity.

We may work to offer a greater range of therapeutic choices to treat a variety of clinical diseases with continued research of probiotic application in veterinary medicine. Finding alternative natural compounds that can improve individual's health is also a crucial topic given current problems like antibiotic resistance.

In this work, the focus was on three bacteria: *Bacillus subtilis, Bacillus licheniformis* and *Bacillus pumilus.* By administrating beta-lactam antibiotics, more specifically amoxicillin, and 3rd generation cephalosporin; cefquinome and ceftiofur, we could observe the phenotypic changes by the help of RNA-isolation and PCR.

The results showed diverse reactions of two of the probiotics to all three antibiotics. *B. pumilus* did not exhibit any significant changes in resistance gene expression under antibiotic exposure. However, *B. subtilis* and *B. licheniformis* gave other results. Our investigation is indicating that certain probiotic strains can develop enhanced resistance phenotypes in response to environmental antibiotic exposure.

B. subtilis with its upregulation of gene *ypxl*, and *PenP,* and *B. licheniformis* of gene *PenP*. These results suggest therefore that resistance can be built up when the *Bacillus* bacteria mentioned above is introduced to the specific antibiotics in the environment, resulting in no, or little effect once the medicine is administered. However, this itself is not necessarily an issue, considering they are probiotics and wanted bacteria. It can even have a positive effect, meaning the pathogenic bacteria will be neutralised by the antibiotics, and the probiotics will remain, stabilizing a healthy environment.

The question that needs to be asked and discussed is if these resistant probiotics can transfer their own genes to the pathogenic bacteria, making them resistant. The study's findings of the upregulation of beta-lactamase genes are consistent with previous research showing that *Bacillus* species may adapt to antibiotics. Probiotics may carry and express resistance genes, according to similar findings, which emphasize the need for caution when using these bacteria in settings where antibiotics are present. Moreover, depending on the situation, these *Bacillus* strains' capacity to survive antibiotic stress may present both benefits and drawbacks.

Probiotics are administered frequently and mixed with the normal microbiota while transferring their resistant genes to the other physiological bacteria that are already there. Unfortunately, there has already been reported transferring of plasmids, transposons, and genetic information between inter- and intraspecies to pathogenic bacteria [6].

With the beta-lactamases´ ability to hydrolyse the beta-lactam ring of the antibiotics, and the variation of the targeted types of antibiotics, these types of studies are crucial to get a better overview. This also applies to the knowledge of genes and enzymes; genes, holding the "blueprints" for the functional proteins, enzymes, to carry out the mechanism leading to antimicrobial resistance. In other words, this is where resistance is created, and therefore, where it can be prevented [23].

Even though *FtsH* can be found in most of bacteria, primarily as a housekeeping protease, it still has the ability to alter the membrane proteins, hence influencing antibiotic resistance. Additionally, during antibiotic exposure, it might establish stress-response pathways due to its proteolytic activity [24, 25].

PenP being found in both probiotics and commensal bacteria, has a significant increased risk of it being horizontal transferred between the bacteria themselves, as well to pathological ones [23, 26].

RepN in directly involved in the microbial resistance propagation due to its role in plasmid replication. By protecting the plasmids throughout the processes, they are making sure the plasmids are brought along with the horizontal transfer [27, 28].

Due to its lack of research of the *ypxl* gene, it is obvious that more studies are needed. In the *Bacillus* genus several class D beta-lactamase were identified, and these studies have shown that all of these enzymes possess some level of β-lactamase activity [44].

All of these genes highlight the environmental adaptability and the complexity of survival strategies in bacteria, both directly and indirectly supporting antibiotic resistance. The genes hold such an immense role in both clinical and environmental microbiology [23].

Some examples on how to handle antibiotic resistance by probiotics would be to check the genotypes and phenotypes of antibiotic resistance in all probiotic strains, and further examine each phenotypic resistance that deviates from the species' standards. Despite the modest danger, the possibility of DNA transmission via transformation calls for more research. Manufacturers of older strains who might not have used this method of assessing the risk of antibiotic resistance ought to re-evaluate their strains to ensure compliance [45].

The administration of the probiotics to provide a healthy vaginal microflora might not be as easy as thought. By having most commonly an oral administration, the probiotics would

mainly affect the gastrointestinal tract, and not necessarily have any large effect of the reproductive tract. There is not done enough research on other ways to administer to say for sure that oral is the most effective. From previous research that has had positive results with both intra-uterine infusion and intra-vaginal pessaries should be further explored [34].

The One Health approach as it emphasizes the close network of humans, animals and environmental health is crucial to keep in mind when dealing with both antibiotics and probiotics. The government of each country, and continents have an important role of implementing policies and frameworks on how and when to administer. During the implementation of these, One Health which focuses on zoonotic disease regarding antibiotic resistance should be taken greatly into consideration [42].

It is also an increased risk of humans receiving antibiotic resistance by the keeping of animals as well as working in the farms and with the production. We can as well gain resistant genes from residues in meat products. Meaning we should take this information seriously and introduce new and more strict regulations for the usage of probiotics before it escalates to a level where antibiotics no longer effective [6].

This study offers valuable insights about the gene expression dynamics of the *Bacillus* species when exposed to antibiotics. By providing accurate assessments of resistance gene upregulation, quantitative PCR allowed us to better comprehend these bacteria's biological reactions. However, the lack of protein-level validation limits the ability to confirm the functional impact of the genetic changes. Proteomic analyses should be used in future research to determine whether the observed genetic upregulation translates to increased resistance at protein level. Furthermore, *in vivo* research may shed further light on how these probiotics behave within complex microbial ecosystems.

Future studies should investigate the circumstances in which these genes could be passed on to harmful bacteria considering the resistance gene upregulation that has been seen. Investigating the environmental and microbial factors that facilitate such gene transfer will be critically important. Moreover, research aimed on development of probiotics with reduced resistance gene content, or increased regulation of their usage, may help reduce the hazards connected to probiotic therapy.

In conclusion, we demonstrated, in *Bacillus* probiotics candidates, upregulation of resistance-coding genes' expressions in some instances, which can lead to decreased sensitivity to certain antimicrobial drugs. Although, our results should be supported by protein measurements in the future, so we can see if the amount of resistance proteins is indeed enhanced in these probiotic strains after incubation with antimicrobial substances.

7. Summary

During recent years there has been increasing focus on our own and others health, increasing the usage of probiotics. These substances stabilize the gastrointestinal microbiome as well as the reproductive tract microbiota of several animal species, and in this case, dairy cows. Reproductive health is crucial for a well-performing industry and the lack of it can lead to detrimental effect of both production and the economy. Mastitis and metritis are the two most frequently occurring disease conditions in dairy cows and they are treated with antibiotics. Because of the frequent application of antibiotics, resistance can be built up and disturb the microbiota and internal environment.

To stabilize the microbiota and maintain a strong immune system, probiotics are often used. They are considered to be one of the best options to reduce the incidence of antimicrobial resistance instead of giving antibiotics, as well as maintaining the natural microbiological environment during the administration of antibiotics.

Probiotic bacterial strains employed in both human and animal use, though, also run the danger of propagating antibiotic resistance genes themselves. In case of the probiotic germs expressing these specific genes, they have the possibility to transfer them to pathogenic bacteria, making antibiotics clinically ineffective. Therefore, our experiments aimed to monitor the antibiotic resistance status of selected probiotic candidate bacteria.

Broth microdilution susceptibility testing was performed using three different beta-lactam antibiotics. The gene expression of beta-lactamase resistance genes in three different *Bacillus* species was monitored via quantitative PCR analysis.

Our results suggest that the 0.5 mg/l amoxicillin treatment upregulated the *PenP* and the *ybxI* genes in *B. licheniformis* and *B. subtilis*. The 0.05 mg/l cefquinome treatment upregulated only the *PenP* genes in *B. licheniformis* and *B. subtilis*. Hence these are phenotypic changes, they suggest that after these bacteria meet with these antibiotics in their environment, they would become more resistant to them.

In conclusion, according to our current knowledge even though probiotics help combat the antibiotic resistance today, they might result in some unfavourable issues for the future. We must think about the '*one health*', for that, different approach might be necessary. Because of both advantageous and disadvantageous concerns, it would be best, even today, to implement proper regulation, not only to livestock, but human usage as well.

8. Összefoglaló

Az elmúlt években még nagyobb hangsúlyt fektetünk saját és a környezetünkben élők egészségére, ami a probiotikumok egyre elterjedtebb alkalmazását vonja maga után. Ezek az anyagok stabilizálják a gasztrointesztinális mikrobiomot, valamint számos állatfaj, úgy, mint a tejelő tehenek szaporodási szervének mikrobiótáját. A reproduktív egészség létfontosságú egy jól működő tehenészet számára, ennek hiánya a termelésre és a gazdaságosságra egyaránt káros hatással lehet. A tejelő teheneknél a tőgy- és a méhgyulladás a két leggyakrabban előforduló betegség, ezeket rutinszerűen antibiotikumokkal kezelik, amelyek gyakori alkalmazása miatt rezisztencia alakulhat ki, ami a mikrobiótát és a szervezetet is károsíthatja.

A probiotikumokat gyakran alkalmazzák az erős immunrendszer megőrzésére. Az egyik legjobb lehetőségnek tartják a fertőzések megelőzésére történő alkalmazásukat az antibiotikumok adása helyett, ezzel is csökkentve az antimikrobiális rezisztencia előfordulását, valamint a természetes mikrobiológiai környezet fenntartását.

Az emberi és állati felhasználás során egyaránt alkalmazott probiotikus baktériumtörzsek azonban antibiotikum-rezisztencia gének terjedésének veszélyét hordozhatják magukban. Az ezeket a specifikus géneket hordozó probiotikus csírák esetében lehetőségük van azokat patogén baktériumokba átvinni, így egyes antibiotikumok klinikailag hatástalanok lehetnek. Ezért kísérleteink célja a kiválasztott probiotikus-jelölt baktériumok antibiotikum rezisztenciájának monitorozása volt.

Mikrohígításos érzékenységi vizsgálatot végeztük levestáptalajon, három különböző béta-laktám antibiotikummal. A béta-laktamáz rezisztencia gének expresszióját három különböző *Bacillus* fajban kvantitatív PCR analízissel követtük nyomon.

Eredményeink azt mutatják, hogy a 0,5 mg/l amoxicillin kezelés a *PenP* és az *ybxI* gének expresszióját *B. licheniformis* és *B. subtilis* esetében fokozta. A 0,05 mg/l cefkvinom kezelés a *B. licheniformis* és *B. subtilis* esetében csak a *PenP gén* aktivitását növelte. Ezek a fenotípusos változások, azt jelzik, hogy miután ezek a baktériumok találkoznak ezekkel az antibiotikumokkal a környezetükben, kevésbé érzékennyé válhatnak velük szemben.

Mindezek alapján, annak ellenére, hogy a probiotikumok jelenlegi ismereteink szerint bár segítenek az antibiotikum-rezisztencia leküzdésében, alkalmazásuk a jövőben akár kedvezőtlen hatásokhoz is vezethet. Az ’*egy az egészség*’ égisze alatt kell gondolkodnunk, emiatt másmilyen megközelítés lehet szükséges. Mind az előnyös, mind a hátrányos szempontok miatt a legjobb lenne már ma is megfelelő szabályrendszert felállítani, nemcsak az állatállományokon, hanem az embereken történő felhasználással kapcsolatosan is.

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Thesis progress report for veterinary students

Name of student: Kristin Skarsbo……

Neptun code of the student:… BP0U7Y……

Name and title of the supervisor: Dr Orsolya Palócz……

Department of Pharmacology and Toxicology……

Thesis title: Gene expression status of beta-lactam resistance in certain probiotic bacteria ……

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	02	10	Culturing of bacteria	
2.	2023	03	17	Experiment on the cultures	
3.	2023	04	21	Sample processing	
4.	2023	05	5	PCR measurement	
5.	2023	05	19	Data evaluation	

Grade achieved at the end of the first semester: ……5 (excellent)……..

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	08	09	Supervision of literature review	
2.	2023	08	29	Materials and methods section	
3.	2023	09	14	Results and summary	
4.	2023	10	24	Discussion section	
5.	2024	11	20	Review of the whole work	

Grade achieved at the end of the second semester: ...5 (excellent).....

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence.

.....
signature of the supervisor

Signature of the student: Kristin Skarsbø

Signature of the secretary of the department: .....

Date of handing the thesis in..... .....