Outline of the Obligatory Annex to the Strategic Research Agenda of the Centre

Annex No. 5 - Strategic outlook and prospects in the field Ref. No. TACR/11-59/2021

1. Vision or brief objective of the project

1.1. Project goal

The aim of the project National Centre for Biotechnology in Veterinary Medicine (NaCeBiVet) is to create a stable and long-term base of applied research in the field of biotechnologies in veterinary medicine, animal production and related fields. The agriculture industry contributes about 2% to the gross domestic product and employs approximately 100,000 workers. Agriculture is mainly a producer of food for the population. In addition, agriculture has a number of other functions - it provides employment in rural areas to a certain extent and is thus part of their sustainability and development. It also has a landscape function - from arable farming and grazing land management to forest management and water resource management. However, agriculture is also considered a source of environmental pollution - from the use of pesticides for plant protection, through the use of antimicrobials for keeping farmed animals healthy, to the production of greenhouse gases.

The NaCeBiVet Centre therefore responds to the socio-political requirements associated with ensuring the sustainable production of quality and safe food of animal origin in terms of implementing policies to reduce antibiotic consumption, improve the welfare of farm animals, maintain biodiversity in the landscape and the principles of circular economy. The results generated in the project in the form of new or innovative products or services will also support the competitiveness of the companies involved.

1.2. Socio-political framework of project implementation

The Centre's goal and planned activities are in accordance with the objectives of the United Nations document entitled Transforming our World: The 2030 Agenda for Sustainable Development, which was officially approved by the UN Summit on 25 September 2015. The document contains 17 Sustainable Development Goals. One of these is Goal 2: "End hunger, achieve food security and improved nutrition and promote sustainable agriculture", in which Target 2.4 aims at ensuring/establishing sustainable food production systems and resilient agricultural practices. Goal 12 "Ensure sustainable consumption and production patterns" in its Target 12.2 describes the need to achieve sustainable management and efficient use of natural resources, Target 12.4 defines the need to achieve the environmentally sound management of chemicals and wastes and Target 12.5 details the need to reduce waste generation.

In order to achieve the long-term sustainability of producing sufficient quantities of good quality and safe food, all actors in the production chain must be willing and able to change their production methods. The

use of innovative technologies will lead to a reduction (or rather rationalisation) of all potentially negative impacts associated with agricultural activities. This is one of the pillars of European Union legislation - the European Green Deal. All 27 EU Member States made a commitment to transform Europe into the first climate-neutral continent by 2050. The Green Deal policy is implemented by packages of proposals to transform segments of the economy. Most important for the National Centre for Biotechnology in Veterinary Medicine project is the "Farm to fork" strategy for a fair, healthy and environmentally-friendly food systems, which was communicated by the European Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions in May 2020. This document states that "Even though the EU's transition to sustainable food systems has already started in many areas, food systems remain one of the key drivers of climate change and environmental degradation. There is an urgent need to reduce dependency on pesticides and antimicrobials, reduce excess fertilisation, increase organic farming, improve animal welfare and reverse biodiversity loss".

The same document also defines the role of research and development in this area. It states that research and innovation will play a central role in accelerating the transformation towards sustainable, healthy and inclusive food systems from primary production to consumption. It also states that the key research areas will include the microbiome, ocean food, urban food procurement, as well as increasing the availability and quantity of alternative protein sources such as plant, microbial, marine and insect proteins. These objectives are consistent with another document through which the Green Deal policy will be implemented: Proposal for a Regulation of the European Parliament and of the Council on the making available on the Union market as well as export from the Union of certain commodities and products associated with deforestation and forest degradation and repealing Regulation (EU) No 995/2010. This document from November 2021 aims to minimise consumption of products coming from supply chains associated with deforestation or forest degradation. Examples of commodities the production of which is linked to deforestation, particularly in South America, include soy and beef.

The topic of the National Centre for Biotechnology in Veterinary Medicine project is also in compliance with documents of the Czech Republic. One of them is the National Research and Innovation Strategy for Smart Specialization of the Czech Republic 2021-2027 (RIS3). The National RIS3 Strategy directs the support towards the selected priority areas that have a high potential for creating a long-term competitive advantage of the Czech Republic based on the utilisation of knowledge and innovation. The document has an internal structure based on vertical and horizontal priorities. The research and application specialisation domain Green Technology, Bioeconomy and Sustainable Food Resources falls under the national innovation platform Sustainable agriculture and environmental industries. It is part of the application sector Natural Resource Management, Agriculture and Forestry, Food Production, Environment and Biodiversity, Construction and Human Settlements. According to this material, biotechnology has the highest potential for using R&D results.

Another document is the Strategy of the Ministry of Agriculture of the Czech Republic with outlook to 2030. According to this document, the long-term vision of the Ministry of Agriculture is the competitiveness and sustainability of Czech agriculture, food industry, forestry and water management. In accordance with the fulfilled vision, the Czech Republic in 2030 is food self-sufficient in basic commodities that can be produced

in the Czech Republic: food production from domestic sources, with a certain degree of independence from the purchase of these foods from other countries. The practical implementation of the Strategy is elaborated in the Concept of Research, Development and Innovation of the Ministry of Agriculture for 2023-2032. The concept is divided into three key areas: (1) bioeconomy, (2) smart agriculture and (3) global changes in the biosphere. The concept defines the following research weaknesses in the agriculture sector:

Very little competition and very little collaboration in research at the same time.

Insufficient feedback on publicly available research results from end-users.

Insufficient practical application of research results in the agriculture-food industry sector, forestry and water management, both in the private and public spheres.

Insufficient private sector involvement in co-funding and funding of research in the agriculture-food industry sector, forestry and water management.

Unbalanced level of research in priority topics and low level of multidisciplinary approach.

Excessive focus on short-term problems and very little attention to long-term issues with a horizon of 10 to 20 years. Absence of long-term projects (5 years or more).

Uncertainty in funding continuity destabilises HR policy of the research organisation and limits the involvement and retention of specialists in some fields.

All these weak points are considered by the project of the National Centre for Biotechnology in Veterinary Medicine NaCeBiVet. The potential significance of the project for the needs of state administration bodies is evidenced by a support letters from the management of the State Veterinary Administration and Central Institute for Supervising and Testing in Agriculture, which are placed among the annexes to the project.

1.3. Agriculture as a food producer

As already mentioned, agriculture accounts for 2% of the Czech Republic's gross domestic product. This is relatively little when compared to the services sector and the industrial sector (especially the automotive industry and related subcontractors). But food production is an indisputable role for agriculture. The economy of agricultural production is based on long-term sustainability. It cannot be turned on and off from day to day. The economy of livestock breeding is also significantly dependent on supplier-customer relations, input prices, international situation, tradability of production, subsidies. Although the Czech Republic is part of the European Economic Area and the Schengen area, which guarantees the free movement of people, goods and capital, the terms local food production and shortening the production chain are increasingly used. Partly in connection with the implementation of the above documents, but also, for example, in an effort to reduce the carbon footprint. The importance of local agricultural production is also underlined by the circumstances of the recent past - the coronavirus crisis and the associated limited global movement of

goods, but also the crisis in Ukraine and the associated concern about sufficient grain production and rising commodity prices.

Increasing the ability to produce food will in part increase society's resilience to such crises. In addition, in some commodities, production in the Czech Republic does not cover consumption for a long time. According to data from the Czech Statistical Office, for example, self-sufficiency in pork production in 2020 was 43.2%. For comparison, in 1998 it was at the level of 98.3%. The situation is similar in the production of poultry meat (59.8 vs. 96.1) or bee honey (74.1 vs. 116.2). On the other hand, beef and milk production is sufficient. Here, however, there is a need to further intensify production to maintain the same production with fewer animals.

The planned activities of the NaCeBiVet project are aimed at all these general goals. The thematic focus can be divided into 4 research directions:

1) Biotechnology in prevention and therapy

2) Biotechnology in diagnostics

3) Biotechnology in nutrition

4) Biotechnology in reproduction

It is clear that this division is rather formal. E.g. good nutrition and the use of probiotics is part of prevention and sometimes has a supportive therapeutic effect. Likewise, diagnostic procedures are the basis for setting preventive measures and, in a broader context, reproduction based on the breeding of healthy animals also leads to a reduction in antibiotic consumption. This division of activities into research directions is based mainly on different methodological and technological approaches to the solution. And in the following text they will be described separately.

2. Current situation in the area/field with an emphasis on future development and trends

2.1. Biotechnology in prevention and therapy

As in any industry, profitability is essential for successful livestock farming. One of the factors significantly affecting the profitability of livestock farming are animal diseases, especially infectious diseases. As in human medicine, the prevention of diseases is far more beneficial than potential treatment. Not only because of the treatment cost, but in the case of livestock farming also because of poor animal welfare, reduced performance or reduced product quality. If a situation arises where treatment is necessary, it must be targeted and effective.

There are three types of pathogens that cause infectious diseases - viruses, bacteria and parasites. Many diseases do not occur in the Czech Republic for a long time. E.g. bovine tuberculosis, leukosis, foot-and-mouth disease. Newly, these diseases include bovine viral diarrhea. These diseases were eradicated by the use of diagnostic methods and the elimination of positive animals and subsequent methods of control of the territory, respectively, stable. The second method was the use of diagnostics in combination with active immunization as a tool for preventive measures.

Nevertheless, there are diseases in animal husbandry in the Czech Republic that negatively affect the economics of breeding or increase the administration of antibiotics. Moreover, for many diseases, especially of viral origin, there are practically no effective therapeutic options. For them, the importance of preventive measures is even more pronounced. Below, there are some examples of infectious diseases that still exist in the Czech Republic and pose a problem for various reasons. Preventive/therapeutical measures against some of them could be subject of particular sub-projects.

Streptococcus suis is currently considered economically and medically most important bacterial pathogen of pigs and its importance has an increasing tendency. A common approach to reduce the transmission of S. suis infection from sows to newborn piglets is the prophylactic administration of antibiotics to piglets shortly after birth. However, this approach is incompatible with the current efforts to restrict the preventive administration of antibiotics in farm animal herds in order to combat antimicrobial resistance in animal and human pathogens. Vaccination of sows against S. suis could be an approach potentially replacing preventive antibiotic treatment. Effective levels of vaccine-induced antibodies in sows could reduce the shedding of S. suis, and antibodies passively acquired by piglets via colostrum and milk should make piglets less susceptible to infection. A problem with vaccination against streptococcal infections in general, including S. suis infection, is the lack of efficacy of conventionally produced vaccines by inactivating the bacterial culture and combining it with adjuvants. Nevertheless, significant progress has been made in vaccination against streptococcal infections with the introduction of the Prevenar 13 vaccine against S. pneumoniae infection in humans. This vaccine uses an innovative approach in the construction of the vaccine antigen. Experimental vaccine against S. suis based on an affordable approach comparable with that of the Prevenar 13 vaccine has been develop and constructed. The aim of this subproject could be to forward the product to practical realization and to develop this vaccine against other important pathogenic serotypes of S. suis, to test its efficacy against these serotypes and, in case of good efficacy, to register it.

Another example of important pathogen of pigs is rotavirus, the causative agent of acute gastroenteritis in suckling and weaned piglets. Rotavirus A infections in pigs occur worldwide with a prevalence between 3.3% and 67.3%. The most common genotypes in pigs are G3, G4, G5, G9 and G11, usually in association with P[5], P[6], P[7], P[13] and P[28]. Rotavirus B was first described in the 1980s, and studies suggest its high variability and a high prevalence in herds worldwide. Rotavirus C has been detected in diarrhoeic piglets in most countries of the world. Other groups of rotaviruses occurring in pigs are rotaviruses E and H, the occurrence of which in the Czech Republic is completely unknown. Vaccination strategies are primarily aimed at enhancing lactogenic immunity, as newborn piglets are not capable of a sufficient immune response. The success of rotavirus vaccines for pregnant sows depends mainly on the strain contained in the vaccine. Frequent administration of the existing vaccines may lead to the gradual replacement of current genotypes

with new ones against which herd immunity is very low. The aim will be detailed characterization of rotavirus isolates in the Czech Republic and their adaptation to cell lines or primary porcine cell cultures. The main result of the project would be culturable and at the same time in-depth genetically characterized rotavirus strains for vaccine production by a partner company.

In cattle, bacterial infections frequently occur in early postpartum period. Antimicrobial drugs in dairy cattle are most commonly used to treat postpartum uterine diseases (metritis) and mammary gland infections in dairy cows. The antibacterial effects of the common sage (*Salvia officinalis*) and the true Iceland lichen (*Cetraria islandica*) can be used in the prevention and therapy of metritis, and we expect their synergistic effect. The antibacterial activity of the active substances against both Gram-positive and Gram-negative bacteria has been described for sage. Essential oil, tannis katechines, bitter substance picrosalvin and lactone Salvin and their fractions have a significant antibacterial effect, including against *S. aureus* a *B. subtilis*, which are among the main causative agents of metritis in dairy cows. The aim will be to develop a formulation for the prevention and therapy of metritis, to determine its efficacy and safety. If its efficacy is good, the registration of the product will be followed by its production by a partner company.

Mammary gland infections - mastitis - limit the usability of the milk produced due to the deterioration of their biological properties, an increase of the number of bacteria and even due to the withdrawal period after the administration of antibiotics. The prevention and treatment of mastitis in cattle accounts for 40% of the total consumption of antibiotics in cattle farms. In order to limit the spread of antimicrobial resistance, the requirement for the lowest possible consumption of antimicrobials is currently being increasingly promoted worldwide. In dairy cattle herds, this means improving mastitis control, including refining their diagnosis, specifying and innovating of procedures for the effective and justified use of antimicrobials for the treatment of clinical mastitis and other common diseases, and promoting good drying-off practices. Streptococci, especially *S. uberis*, which is the most commonly detected mammary pathogen next to coagulase-negative staphylococci, mainly contribute to antimicrobial consumption. No serotypes have yet been described for *S. uberis*, although pathogenic and non-pathogenic serotypes can be expected based on the analogy with other streptococcal species. By analysing the whole-genome sequences of S. uberis, we assume the occurrence of at least 20 different serotypes in the Czech Republic. The introduction of S. uberis serotyping can be expected to significantly increase knowledge of the epidemiology of this pathogen. Based on the experience with the creation of software tools for cattle health status monitoring (Disease and Treatment Diary application, user ČMSCH a.s.), software tools will be developed for an effective procedure of selective drying-off practices and a specialized professional database, both of which will be used by dairy cattle breeders and cooperating companies in their consulting. Moreover, a certified methodology for serotyping of S. uberis, which will be applicable at the State Veterinary Institutes for the detection of causative agents of mastitis could be expected output of this sub-project.

Rabbit industry is also affected by infections diseases. Viral pathogens relevant for rabbits include myxomavirus inducing myxomatosis and calicivirus associated with so called rabbit haemorrhagic disease. Against both of them, there are commercial vaccines available on the market. Their production, however, is associated with use of rabbit to produce the virus that is used in the vaccine. With line of 3R (reduction,

replacement and refinement) policy, there is need to change the production approach. One of the way it to create vaccine based on another technology – for example, virus-like particles.

The last type of pathogens is represented by parasites. External and internal parasites are among the most common livestock pathogens in Europe with a significant economic impact on livestock production. The apparent trend towards improved animal welfare is closely linked to the expansion of ruminant grazing farms or the increase in the share of extensive pig and poultry farming. As a result, the parasitic burden on grazing animals or animals with an outdoor enclosure increases. The only solution to this problem is a sustainable anti-parasitic program for a specific species of livestock. The use of antiparasitics in livestock poses three major problems: 1) the emergence and spread of resistance to available antiparasitics, 2) the risk of antiparasitic residues in products of animal origin from treated animals and 3) negative effects on non-target organisms during antiparasitic therapy (ecotoxicological effect medicines).

Given the enormous length of development (15-20 years) of new active substances with antiparasitic effect, the range of available antiparasitic drugs for livestock is very limited and emphasis is therefore placed on more effective use of already used therapeutics. Avermectins, milbemycins and spinosins are substances belonging to the group called macrocyclic lactones (ML). These are compounds discovered in the last 20 years of the 20th century and are currently one of the most widely used antiparasitic agents in animals. Unlike other chemotherapeutics such as benzimidazoles or levamisole, they are characterized by acting against both internal helminths and a number of ectoparasites, including parasitic species of mites and insects. There are a number of medicines on the market with ML or in combination with other substances that are widely used in ruminants, horses, pigs and pet animals. In addition to the well-known problem of growing resistance to ML, the environmental impact of ML on non-target grassland organisms remains neglected. There is ample evidence of an inhibitory effect on ML on coprophagous beetles or other insects in animal pastures. The massive use of ML in grazing animals thus negatively affects biodiversity in the landscape and has an indirect impact on soil quality. One of the key factors is the low bioavailability of ML resulting in high excretion of ML from treated animals into the environment. It is reported that up to 90% of an administered dose of orally administered ML is released into the environment.

The issue of residues in food of animal origin concerns not only hormonal and antibiotic preparations, but also antiparasitic drugs. Many very effective veterinary antiparasitic drugs must not be used in dairy cattle whose milk is intended for human consumption. This is either due to the transfer of the substance to milk or to the lack of information on maximum residue limits (MRLs) for milk for a given animal species. In practice, it happens that lactating cows, sheep and goats have nothing to deworm.

Advances in the pharmaceutical industry have been brought about by the use of nanotechnologies, in particular the application of nanoparticle delivery systems for drugs. These nanoparticle delivery systems can also be used to increase the bioavailability of water-insoluble macrocyclic lactones. By preparing nanoparticulate delivery formulations of antiparasitics, we can increase their bioavailability in the body, reduce the dose and at the same time reduce the excretion of the substance into the environment. The new original ML formulation prepared in this sub-project will contribute to higher drug efficacy and lower negative environmental impacts of the drug.

2.2. Biotechnology in diagnostics

Timely and correct identification of the pathogenic microorganism is a critical prerequisite for the application of effective preventive, eradication or therapeutic procedures in all areas of farm animal production. In case a bacterial pathogen is detected, it is necessary to select an appropriate antimicrobial agent for potential therapy due to a certain level of resistance to antimicrobials possessed by various pathogenic bacteria and the limitation of further increase in their resistance. Along with the increasing need for effective diagnostics, the need to introduce innovative molecular biological procedures into the veterinary medicine environment is also growing. In the field of veterinary medicine, point-of-care (POC) and point-of-need (PON) applications are simple to use, portable, easily disposable, and stable under different operating conditions. Though many methods are already established for veterinary diagnostics, not all of them are suitable for point-of-care diagnostics. In the area of molecular diagnostics nucleic acid amplification methods are very sensitive and specific due to target amplification and base-pairing interactions. Over polymerase chain reaction (PCR), the isothermal amplification of DNA / RNA has recently drawn interest since it does not require a large thermal cycler. Unlike the standard PCR, LAMP methodology does not need a molecular biologist or other specialists to run it. The Loop Mediated Isothermal Amplification (LAMP) method allows the amplification of nucleic acid (DNA / RNA) under isothermal conditions at a constant temperature, under much simpler conditions at a faster time. Moreover, LAMP considered as a robust method in terms of sensitivity, tolerance to inhibitory substances present in the real sample, and enables the visual detection. Therefore, it is a simpler and more energy efficient approach, making it an excellent choice for POC applications.

The carp fishing industry in the Czech Republic has been struggling with economic losses due to infectious diseases of viral origin for a long time. There is currently no specific treatment or a registered vaccine against common carp viruses, so prevention and eradication of outbreaks are the only tools to combat economic losses. Prevention is primarily based on the protection of farms against the introduction of pathogens, mainly through quarantine of newly purchased fish and diagnosis of infectious diseases if disease signs appear. On the other hand, once an outbreak in the case of a notifiable disease (KHV) is confirmed, emergency veterinary measures (ban on transport of fish and their products, depopulation, draining of tanks and disinfection of their bottom) are declared in the case of notifiable diseases (KHV). Both these strategies rely on sensitive and specific detection of the presence of viral pathogens. This is currently based mainly on molecular (PCR) and virological (culture on a specific cell line) methods. Both are time-consuming and require transport of samples to an adequately equipped laboratory. A rapid test for KHV, produced in Austria, based on an immunochromatographic strip, is currently available on the market, but methods for rapid detection of CEV based on antigen detection are not yet available even in the literature. For pathogen detection under field conditions, the LAMP method by using direct detection of the pathogen nucleic acid without the need for complex laboratory equipment, appears promising. The development of diagnostic tools using the LAMP method is well established at the department. The LAMP method will be used to develop kits for the detection of common carp viruses.

Winter losses of bee colonies have been steadily increasing since 2007. The situation has escalated several times since then, with widespread collapse of bee colonies in many places in the Czech Republic. Although research in this area has produced a number of partial findings, particularly in the field of bee viral diseases,

it has not been possible to fully elucidate the broader context, let alone the causes. This situation is most commonly associated with the varroa mite (*Varroa destructor*), which parasitises both brood and adult bees. This mite is the main vector of a number of viruses, in particular Deformed wing virus (DWV), to which the collapse of honey bee colonies has been linked worldwide in recent years. Previous studies confirm that the mite is able to accelerate virus replication and modulates the bee's immune system. In collaboration with partners from Mendel University, we will focus on the development of diagnostic methods for routine diagnosis of selected viral infections usable under field conditions, including sample preparation procedures. Systems for the diagnosis of viral bee paralyses (acute bee paralysis, chronic bee paralysis) and infections of the brood of honey bee (brood pox virus, possibly Black queen cell virus, *Paenibacillus larvae* - the causative agent of brood plague and *Melissococcus plutonius* - the causative agent of brood rot) will be developed and validated. The LAMP method will be used for the detection of honey bee pathogens under field conditions.

In the field of veterinary medicine, point-of-care (POC) and point-of-need (PON) applications are simple to use, portable, easily disposable, and stable under different operating conditions. Though many methods are already established for veterinary diagnostics, not all of them are suitable for point-of-care diagnostics. In the area of molecular diagnostics nucleic acid amplification methods are very sensitive and specific due to target amplification and base-pairing interactions. Over polymerase chain reaction (PCR), the isothermal amplification of DNA / RNA has recently drawn interest since it does not require a large thermal cycler. Unlike the standard PCR, LAMP methodology does not need a molecular biologist or other specialists to run it.

Current trend in the accommodating of LAMP method into veterinary laboratory or field practice requires the development in i) testing protocols for diagnostic cases and in second ii) instrumentations. The complexity of the instrumentation is then based on the requirements of each protocol.

Most simple protocols are useful for viral detection in matrix, containing high viral dose. Due to the Covid19 pandemic, this case is now well developed and ready for use not only in human diagnostics for „sample to answer“ basis.

The challenge for development of protocols remains to be addressed for:

i) samples with low viral load with necessity of preconcentration,

ii) for more complex samples where matrix effect can interfere the LAMP components to work properly,

iii) for bacteria detection in highly diluted samples.

In the initial phase of projects, it is usually necessary to optimize the basic protocol, procedures and reaction conditions. It is mainly about determining the optimal reaction temperature, testing the most suitable primer sets for the target sequence of the pathogen and selected internal controls. In some cases, the preconcentration and cleaning steps are required. For such steps the magnetic particles could be used and applied much conveniently then in PCR applications.

The LAMP device must be compatible with the selected protocols and at the same time should meet the requirement to perform the LAMP test in field conditions. Lower energy consumption, low weight and robustness of the developed equipment are desirable.

Taken together, LAMP technology is one of the most promising methods of molecular biology applicable to veterinary practice, because it can provide low cost but laboratory precise results in the point of need.

Veterinary medicinal products containing antimicrobials are one of the essential tools used to effectively control infectious bacterial diseases in animals. In recent years, however, not only in human medicine, but also in veterinary medicine, we are beginning to encounter more and more cases of reduced sensitivity or even complete resistance of bacterial pathogens to some antimicrobials. The availability of correct and accurate information on the susceptibility or resistance of a given pathogen thus becomes crucial for the veterinarian when choosing an antimicrobial. It is not only from the point of view of treatment of an individual animal or a group of animals when disease breaks out, but it also helps create a certain strategy of antimicrobial use and health management in a herd from a long-term perspective and is a prerequisite for fulfilling the principle of responsible use of antimicrobials in veterinary medicine, which is and will be increasingly emphasized in the future. In addition, the international context needs to be taken into account, as many EU and non-EU countries are already setting or have set rules for the responsible and prudent use of antimicrobials. This is based on results from a given region mapping the status and trends in antimicrobial resistance. A prerequisite for the possibility of comparing the status and development of resistance trends of bacterial populations to antimicrobial agents in different regions is the use of harmonised procedures for the determination of bacterial susceptibility/resistance to antimicrobial agents by means of diagnostic tools that meet the requirements based on international standardised methodologies (EUCAST, CLSI). The VRI is manufacturer of a kit for the determination of bacterial susceptibility/resistance by a standardized microdilution method. The kit has been currently introduced into the market, but requires storage at -20°C, which significantly limits the possibilities of its distribution, especially in veterinary medicine. A high added value would be the possibility to store it at room temperatures and to extend the expiry date of the product. Therefore, the aim is to develop a lyophilized kit that would have these properties, perform stability tests, and transfer the innovated product to GMP production.

2.3. Biotechnology in nutrition

Legislation of the Czech Republic (The Feed Act No. 91/1996 Coll.,) previously permitted the use of selected antibiotic growth stimulants in animal nutrition. Due to the growing resistance of microorganisms to antibiotics due to their excessive use, the preventive administration of antibiotics and the use of antibiotic growth promoters in food-producing animals have been banned. In accordance with Regulation (EC) No 726/2004 of the European Parliament and of the Council, the use of antibiotics as stimulators of animal growth and performance has been banned in all European Union countries since 2006 (due to the elimination of antibiotic residues from the human food chain). Due to this ban, many scientists are studying alternative approaches to the use of various substances (eg. biologically active substances, prebiotics and/or probiotics, phytogenic additives, micro-elements) with a growth-promoting effect and also improving animal health and

thus food quality and safety. Opportunities are being sought to increase the nutritional value of animal products and follow-on foods through animal feed and nutrition, thus bringing potential health benefits to consumers.

In veterinary medicine, a gradual shift away from infections to the topics connected with reducing the costs and impacts of production, increasing the productivity and reducing antibiotic consumption can be observed in the recent decades. One of the research areas that links all the above topics is improving the quality of nutrition, either by selecting new feed supplements of a prebiotic nature or by preparing new probiotic preparations. A return to natural materials and natural procedures is usually associated with long-term sustainability and low negative environmental impacts. Such topics are perceived positively by society, in contrast to e.g. vaccines, which are effective but have been prepared by genetic manipulation. The use of natural and organic practices improves animal welfare, reduces stress associated with increased susceptibility to infections and as a result, for example, leads to reduced use of antibiotics. The importance of the gut microbiota in improving the well-being of farm animals and humans is therefore one of the topics currently receiving considerable attention. In humans, the relationship between gut microbiota composition and diabetes, obesity, allergies and even autism is gradually being demonstrated. The term gut-brain axis, loosely described as the relationship between gut microbiota composition and host behaviour, is perhaps overrated, but despite of that, or just because of this, it deserves attention. In farm animals, e.g. chicks, it has long been known that a complex microbiota provides protection against Salmonella colonisation.

Fundamental changes have occurred in the structure of the environment and conditions in commercial farm animal production. In commercial production, there is a much higher concentration of animals in one place than in the wild. In laying hens, unisexual flocks of tens of thousands of birds are formed. In poultry, parents and offspring never come into contact so that the microbiota typical of the domestic chicken could be transferred from hens to chicks. The colonisation of chicks in the first days of life is thus entirely dependent on environmental sources, which may or may not be of a suitable composition. The fact that the domestic chicken can live up to 20 years is disregarded and four-week-old broilers are considered to be the reference and near-adult animals.

One of the many problems facing modern broiler chicken production is physiological stress. Under conditions where birds are exposed to stress, supplementation of selenium, as a key enzymatic cofactor, glutathione peroxidase, increases the antioxidant capacity in animals and reduces the harmful effects of free radicals. Selenium added to feed improves animal production and health and has a positive effect on the immune system, quality, content and composition of fatty acids in meat and eggs. Other substances usable both as feed and in meat production can be extracts from plants and their parts also used as food or spices. For example, studies evaluating the effects of Se sources (sodium selenite, seleno methionine, nano seleno methionine) in diet and rosemary essential oil on performance, meat quality, several biochemical and immune parameters of blood and storage in the liver and muscles of broilers showed that the combination of Nano-Se supplements and rosemary essential oil could improve growth parameters, meat quality and immune function in chickens.

Meat quality parameters are formed and influenced by factors that can be defined as intravital or postmortem. The method of breeding has a significant effect on the quality, but also the amount of meat

produced within the carcass. The inclusion of different feeds in animal nutrition has different effects on meat quality. In general, if feed has a positive effect on animal health, it also has a positive effect on meat quality. On the other hand, it must never adversely affect the health of the animal, its use must not be disproportionately unprofitable, and at the same time it must not have a negative effect on the sensory quality of the meat. Of the intravital influences, the current state of animal health is of great importance. For example, heat stress in chickens worsens the quantitative and qualitative parameters of broiler production and affects the quality of meat.

To ensure optimal animal nutrition, it is also important to ensure a balanced intake of structured feeds, which have an impact on animal health and performance. In addition to the quality of feeds and feed materials, their technological adjustment is of fundamental importance. Especially in non-ruminants (poultry, pigs, calves during the dairy period) the most common way of nutrition is to feed complete feed mixtures. In their production, it is important to ensure not only the optimal amount of nutrients in the right proportions, which meets the needs of the species and category of animals, but also their quality, safety and optimal particle size. The production of compound feeds thus requires modern technologies to meet these requirements. When producing compound feeds, it is mainly the possibility of grinding, mixing, "hygienization", application of feed additives, additives, or. medication and other technological modifications of feeds enabling the production of a homogeneous, easily digestible and technologically easily presented feed mixture. Much literature suggests that in the production of compound feeds for animals, a certain uniformity of the individual components should be observed to ensure a homogeneous compound feed. The most important thing in the production of such a feed mixture is to have the highest proportion of medium particle fractions (from about> 0.5 mm to> 1.4 mm). The optimal distribution of the individual particle fractions in the feed mixture will ensure the optimal course of peristalsis, digestibility and nutrient utilization in the animals during the digestion process, and will have a positive effect on the health and performance of the whole organism. In addition, a feed mixture with a low proportion of fine (ie dusty) particles will not endanger production, breeding technologies and, above all, human and animal health.

Modern and sustainable animal nutrition within an animal production, must, in addition to the above, also look for ways to ensure the protein components of feed. Today, the most widely used protein component of feed is soybean extracted meal or fish meal, the prices of which have risen dramatically in recent months. Therefore, the focus is on worldwide research into alternative protein components for animal feed. Possible alternatives are then extracted sunflower meal, lupine seed meal or insect products.

Insect research for possible uses as food and feed is receiving increasing attention worldwide, which has increased the most since 2015 (according to available data in the Web of Science database; van Huis, 2020). It is estimated that the human population is expected to increase to 9 billion by 2050, with an increase in consumption of animal products of 60 to 70%. This increase in food consumption will require huge resources, but are limited by the limited availability of natural resources, continuing climate change and competition between "food-feed-fuel". If the availability of today's most widely used conventional protein feed sources (such as soybean meal and fishmeal) were to be reduced in the future, global competition to ensure sufficient protein for human consumption and production would probably increase. pet and livestock feed. For this

reason, the possibilities of producing alternative and at the same time sustainable protein sources are currently being developed, where insect breeding could be one of the alternative solutions.

The study of microbiota composition of almost any type of sample is relatively trivial due to the technological developments over the last 15 years. However, many researchers entirely overlook the general facts. We have already mentioned the absence of contact between chicks and hens and the difficult to predict intestinal microbiota composition in chicks in the first weeks of life. Nevertheless, papers continue to be published on the time-dependent development of the gut microbiota in chicks, and it is debated whether E. coli must be the first and whether Blautia, Alistipes, or Bacteroides are the next bacteria to colonise the chick gastrointestinal tract. Unfortunately, this is a misunderstanding of the problem and everything is right and wrong at the same time. While all papers of this type correctly identify the sequential colonisation and the first species colonising the chick's caecum in a given study, they fail to recognize the environmental origin of these bacteria. In other words, if the next time Faecalibacterium, Parabacteroides or Barnesiella happens to be present in the animal facility, further papers will appear reporting that not Alistipes but Barnesiella are among the first bacteria to colonise the poultry digestive tract. And yet, it would only be needed to set up a simple experiment in which chicks are placed in the same space as an adult hen, and such an experiment will show that after only 24 hours of contact between the chicks and the hen, the chicks are colonised with adult-type microbiota and become resistant to Salmonella infection.

In pigs, the situation is partly different from poultry, as the contact between sows and piglets has not been broken even in commercial production. However, milk nutrition is very different from the composition of feed for sows, which is reflected in considerable differences in the composition of intestinal microbiota in sows and their piglets. The most typical difference is the presence of the Bacteroides genus members in suckling piglets and the replacement of this genus by related bacteria of the Prevotella genus in pigs of all age categories after weaning. Thus, sows do not always function as a suitable and efficient source of Bacteroides to newborn piglets. Conversely, at the time of weaning, the proportion of Bacteroides is declining and the "demand" for Prevotella and some other bacteria typical of weaned pigs, which are on a standard diet differing from milk nutrition, is increasing.

Therefore, in the production of both chicks and pigs, situations arise where it is appropriate to modulate the composition of the intestinal microbiota so that it is highly likely to correspond to normal development and behaviour. The chick and piglet digestive tract can be deliberately colonised by probiotics in the first days of life, and in piglets and pigs also during weaning. Gut microbiota modulation can also be carried out in adult animals outside these situations, e.g. after an infection associated with antibiotic therapy.

All the above-mentioned facts can be downplayed and described as common knowledge. Current research into the gut microbiota has long focused on characterizing the standard composition and identifying the composition of the gut microbiota typical of individuals that are not developing properly. Alternatively, changes in the intestinal microbiota are monitored in relationship to different compound feed compositions to identify feed supplements that boost desirable bacteria in the intestinal microbiota. Tens to hundreds of papers describing the composition of the intestinal microbiota of chicks and pigs at different age categories, after antibiotic therapy or after experimental increases in the fat (protein, fibre, vitamins, zinc, etc.) content of compound feeds can be documented. They consistently describe that the microbiota of the distal parts of

the digestive tract consists of about one thousand different bacterial species. However, the existing probiotic preparations are limited to a maximum of tens of species belonging to a few genera (Lactobacillus, Bifidobacterium, Enterococcus, Bacillus). This discrepancy is due to the fact that the digestive tract is predominantly colonised by obligate anaerobic bacteria that require specific handling. Nevertheless, the potential of using such bacteria as probiotics is extreme. However, globally, there is a minimum of workplaces dealing with the culture of bacteria from the digestive tract of farm animals and, if they exist, their activities end with publications describing a new bacterial species. To the best of our knowledge, hardly any other laboratory after culture and characterization, e.g. by using whole genome sequencing, continues to test the obtained bacterial isolates as potential probiotics. Experimental work with pure bacterial cultures or defined mixtures of bacteria is the only way to confirm a causal relationship between the presence of a particular bacterium and the host response. The isolation of bacteria from the digestive tract in pure cultures is therefore an absolutely essential step for understanding and subsequent effective application of the findings. The isolation of bacteria in pure cultures is enabled by whole genome sequencing. The whole-genome sequencing technology allows prediction of all protein-coding genes, and knowledge of the amino acid sequence of all proteins allows the use of protein mass spectrometry for the detection of expressed proteins and definition of the most common metabolic pathways of each bacterium. Working with pure cultures allows experimental colonisation of chickens and pigs, and the identification of bacteria, which effectively colonise the gastrointestinal tract after a single application and which, on the contrary, require repeated administration. However, the most important is that no completely new probiotic mixtures can be formulated without pure bacterial cultures. Systematic culture and identification of bacteria colonising the digestive tract of farm animals is therefore essential for any progress towards the practical use of digestive tract bacteria as a new generation of probiotics.

At the time of applying for the National Centre of Competence in Manipulating the Intestinal Microbiota of Poultry and Pigs project support, we verified the functionality of our working hypotheses at the experimental level. First, we compared the composition of the intestinal microbiota of the domestic chickens, domestic pigs and humans. This comparison allowed identification of species that are specifically adapted to some of these hosts and to species that occur in all three hosts. For the development of probiotic preparations, we prefer host-adapted species, because we assume that non-host specific bacteria originate in the environment from which they are continuously supplemented to the digestive tract. We have gradually built up a collection of more than 600 different isolates from the digestive tracts of poultry and pigs. In poultry, we know which bacterial species the hen transmits to the chicks and which are therefore absent from the gut microbiota of chicks in industrial production. In piglets, we know the principles of the development of the intestinal microbiota in the first days of life and at weaning. In chicks, we have experimentally verified that we are able to formulate defined probiotic mixtures that effectively protect chicks from Salmonella infections. However, we encountered new problems in chicks during transfer of laboratory findings to end users. We tested our products in almost 1 million broilers, and although we have mostly observed a positive effect on the health status of the flock, we have also faced situations, and not exceptionally, where higher mortality occurred in the probiotic-treated flock than in the control flock. The reasons are currently unknown, but differences exist between laboratory and field administrations of probiotics. Under laboratory conditions, each chick receives a high dose of probiotics individually. In commercial production, we administer probiotics by spraying the

chicks. Each chick is thus exposed to a lower dose of probiotics, but the administered mixture can also affect the respiratory tract, skin, conjunctivae, auditory canals, etc. The possibility that some of the bacteria common in adult hens behave as opportunistic pathogens in naive chicks cannot be excluded. At the time of the project proposal preparation, we found that two days after the probiotic mixture administration, i.e. at the same time when in some cases increased mortality rates are observed on farms, lactobacilli multiply significantly in the caecum of chicks. In the following days, along with the decreased in mortality, the lactobacilli in the caecum decreased and on the seventh day of life, when we usually check the efficiency of colonisation, lactobacilli constituted only about 0.1% of the total bacterial flora of the caecum of chicks. At this time, we do not know whether overgrowth of lactobacilli in the digestive tract of three-day-old chicks can cause an increase in mortality. Another unresolved issue we noted is connected with the storage and distribution of the product. At the time of the NaCeBiVet project proposal application, we can only store and distribute probiotic mixtures frozen at -70°C. Such storage is sufficient for pilot studies. However, for wider distribution, this method of storage and distribution is complicated and we would need to convert the frozen form of probiotics into a lyophilised form.

In pigs, we can control diarrhoea in newborn piglets by selecting the right mixture. However, as with chicks, we can only store and distribute the probiotic mixture frozen at -70°C. This is logistically unacceptable in the long term and it will be necessary to convert the product into the lyophilised form. In addition, earlier preliminary results show us that lyophilisation of strains for piglets is more feasible than lyophilisation of bacterial isolates from chicks for unclear reasons. Furthermore, we have no mixture for weaned pigs. At the same time, we can define the composition of a suitable mixture for weanlings and we have several suitable isolates in our laboratory collection. Thus, we need to complete the final steps necessary for widespread market application for completing the products and projects under development and, at the same time, we have further plans for the development of completely new probiotic preparations for global animal health protection and the production of safe food of animal origin for humans.

2.4. Biotechnology in reproduction

Cattle farming is globally one of the pillars of producing enough high quality animal-derived food for the growing human population. However, regarding the requirements for reducing the impact of livestock farming on the environment, it is necessary to fulfil the demand for increasing the production by improving the productivity of animals without substantially increasing their numbers. There is continuous pressure to optimise the performance characteristics of dairy cattle and beef cattle breeds. The key solution to achieving this goal is animal breeding followed by rapid introduction of genetically elite subjects into herds. The feasibility of this method has been demonstrated in dairy cattle, whose numbers have shown a steady decline (according to the Czech Statistical Office, 1,247,567 cows were kept in the Czech Republic in 1989 and 585,904 in 2020), even though the total production remained at the same level or increased slightly (the average annual milk yield per cow was 3,982 litres in 1989 and 8,893 litres in 2020). As a consequence, this not only leads to an appreciation of the investment in breeding and the economic profit of the farms, but also has a noticeable effect on the environment, for example on the production of greenhouse gases.

However, a disadvantage of the application of conventional breeding practices is that they are time consuming due to the generation interval of cattle. It has been shown that a possible way to circumvent at least partly the time-consuming breeding process is to introduce biotechnological procedures using germ cell and embryo manipulation and their selection under *in vitro* conditions, allowing faster and more efficient use of elite animals. The breeding objectives can thus be attained within an acceptable timeframe. Assisted Reproductive Technology (ART) is mainly used in these procedures, involving, among others, the *in vivo* recovery of germ cells from animals not yet fully employed in the reproduction process, subsequent *in vitro* embryo production (IVP), evaluation and selection followed by transfer of embryos to recipients. ART methods, originally applied to dairy cattle breeding, are now also used in an integrated system using a combination of dairy and beef cattle breeds. Furthermore, molecular genetics methods are used, in particular the analysis of specific DNA markers of production performance (e.g. SNPs - Single Nucleotide Polymorphisms) and currently also genome sequencing applied to embryo-derived material. By combining ART and genome-based performance testing, it is possible to significantly shorten the generation interval, improve breeding success and increase economic profit of the breeder while reducing the impact on the environment.

As part of the NaCeBiVet activities we intend to focus on the development of methods that are currently used abroad, but are not generally available to our breeders, despite the fact that they could allow them to substantially improve and accelerate breeding programmes. These include, in particular, mastering of gentle collection of germ cells from young age categories of animals, their further manipulation under *in vitro* conditions and subsequent successful transfer to recipients in the form of *in vitro* fertilised embryos. Furthermore, we intend to focus on methods that are being developed abroad, but are not yet routinely applied, and we assume that they have excellent potential and their wider application in the short term can be expected. In our project plans, these methods include micromanipulation of germ cells under *in vitro* conditions (ICSI - Intracytoplasmic Sperm Injection) and 3D embryo culture methods. Last but not least, we intend to focus in our project on the optimisation of methods applicable in routine practice for the evaluation of germ cells or embryos. These methods will allow better characterisation of the quality and potential of biological material for further use.

3. Key objectives in terms of technologies and knowledge that are achievable within 3 years, and at the end of the project

3.1. Biotechnology in prevention and therapy

Objective 1: Development in pig industry

To develop new or innovative vaccines for prevention of currently important infections in pigs in order to improve animal health and decrease antibiotic consumption. Innovative vaccine design used in human medicine was adopted for development of experimental vaccine against pathogenic serotype 2 of Streptococcus suis and one part of this objective will be to further develop this vaccine for other clinically

relevant S. suis serotypes. Similarly, to prevent rotavirus infections in pigs, rotavirus strains currently present in pig populations will be collected, adopted for growth on cell lines a subsequently used for vaccine preparation.

Objective 2: Development in cattle industry

In order to decrease antibiotic consumption for uterine diseases (metritis) treatment a herbal extracts will be used to develop a formulation for the prevention and therapy of metritis. Likewise, improved measures for mastitis prevention have potential to significantly reduce mastitis prevalence and antibiotics consumption for its treatment. In cooperation with with cattle breeders and consulting companies a software tools will be developed for an effective procedure of selective drying-off practices. Innovative approach for Streptococcus uberis typing will be used to monitor pathogenic strains of this prevalent mammary gland pathogen.

Objective 3: Development in rabbit industry

During first three years of the project, new technology will be used for development for a development of experimental vaccine against rabbit myxomavirus and calicivirus. The prototype will be further transferred to pilot-plant conditions. Experimental vaccine will be tested for a safety and efficacy. After potential registration, production will be transferred to full production

Objective 4: Development of antiparasitics

In the first three years, the aim will be to verify the concept of a new formulation of antiparasitic drugs for ruminants. This includes the development, preparation and physico-chemical characterization of a selected macrocyclic lactone nanoemulsion for oral or transdermal administration to small ruminants. Furthermore, in vitro and ex vivo testing of the prepared formulation, pharmacokinetics of the drug in a rat model, testing of antiparasitic efficacy in laboratory rodents. A detailed production procedure in good manufacturing practice will be developed, pharmacokinetics and pharmacodynamics in GLP conditions on target animals (sheep, goats) will be determined, which is an integral basis for registration. Efficacy will be tested on target animals experimentally infected with the most common gastrointestinal and pulmonary nematodes, including resistant and susceptible strains as well as mites. The safety of the new product will be tested on healthy animals in order to evaluate the effect of the therapeutic dose and its multiple on the clinical condition and laboratory parameters in the blood of healthy animals. The project will include the detection of residues in organs, milk, faeces and soil after application to target animals (depletion and environmental assessment). In the following years of the project and at the end of the project, the effectiveness of the new product, including a persistent study, in field farming will be tested. All necessary information will be obtained to complete the marketing authorization of the medicinal product. Certification of utility model and proven technology will be performed.

3.2. Biotechnology in diagnostics

The objective of the project is development and optimization of the LAMP method for the detection of important veterinary pathogens. The subsequent development of LAMP devices is driven by the protocol aim.

Objective 1: Development of the LAMP protocols for G- and G+ bacteria

This key objective is dedicated to multiple levels of the optimization. The most challenging are the preconcentration (microbial enrichment) and lysis steps. Firstly, it is needful to design and optimize the isolation step suitable for preconcentration of bacteria from much diluted samples (such as water, or milk). This might be facilitated using magnetic nano/microparticles with different surface (shell) modifications (e.g. TEOS, APTES) decorated by various recognition molecules (e.g. peptides, antibodies, antibiotics). The next step of protocol is lysis of bacteria where critical point is to maintain the highest level of RNA/DNA yield. Here the thermal, chemical (e.g. chaotropic salts with detergents) or enzymatic (e.g. lysozyme) ways will be tested. The lysate for final LAMP reaction has to be also suitable regarding chemical and physical composition, so adjusting steps might be also researched. The primers design for target bacterial genome will be designed using scientific databases / and or taken from previous testing of other research teams from the NaCeBiVet consortia.

Objective 2: Development of the LAMP protocols for herpetic viruses

Successful protocol for virus detection is commonly dependent on preserving the intact nucleic acids and on lysis and inactivation of the viruses. The composition of lysis buffers for isolation of intact viral RNA/DNA will be optimized. In case of RNA viruses, the RNA once eluted, will undergo by the transcription into cDNA by RT. Amplification of the cDNA will be performed by LAMP and amplicons will be detected. The adjusting of the chemical composition before mixing with the LAMP premix has also to be checked. The target primers for LAMP assay will be designed based on information given by other research teams from the NaCeBiVet consortia based on the target diagnostic application needed.

Objective 3: Adaptation of protocols to a disposable microfluidic chip

The adaptation of LAMP chemical diagnostic protocols in to the microfluidic device is essential for fully automatic point of need (PON) use. The chip structure will be designed according the steps required for full diagnostic operation, with or without magnetic isolation, and based on the sensitivity required with colorimetric or fluorescent readout. Main material platform used will be by CNC drill processed lastic (polypropylene PP-H, or polycarbonate). Then the manipulation with magnetic particles within microfluidic chip will be tested and evaluated. The workflow of in-chip operation will be tested and feedback will be used to redesign the chip. The microfluidic chip will have an optically transparent window where it will be possible to detect the presence and concentration of the amplicons either by colorimetry or fluorescence. The yield and quality of in-chip isolated DNA/RNA will be evaluated.

Objective 4: Design and building of low cost “sample to answer” LAMP PON device

Based on final application requirements such as place of use, overall cost, presence/ use of magnetic isolation steps, sensitivity - type of readout (colorimetric/fluorescence) the final small footprint and low energy consumption device has to be proposed. The Solidworks SW, 3-D printing unit, electronic parts design / assembly will be applied directly within our workplace in the consortia. Some construction parts, fluidic simulations, energy supplies pack etc. might be delivered form the coworkers or as the outsourced service. Finally, the PON device will be ready for one step analysis of one sample with using removable plastic microfluidic chip. The cost of device should be below 50. 000 CZK, cost of microfluidic chip with the LAMP

chemicals should be below 300 CZK, and time of operability of device should be enough to test 10 samples for one battery-capacity cycle (and minimally 48h of standby mode). The signalization of the sample negativity/positivity will be through analogue display. The system will have the simple function of saving of data o last couple of tens samples/measurements. The device will be ready for CE certification meets the RoHS and will be water resistant and shake resistant.

3.3. Biotechnology in nutrition

Objective 1: Microelements and vitamins, nutrition and health of calves / cattle, or poultry

The use of vitamins and microelements in the prevention of diseases in cattle (especially calves) - the main area would be the development of optimization of vitamin E (or other micronutrients) in cattle, respectively. Calves in order to improve their health. It turns out that low levels of vit. E and trace elements are one of the main causes of increased morbidity.

Dairy and starter nutrition of calves in relation to the optimization of health and future performance. Another possible area is the optimization of safe and effective levels and forms of microelements in relation to the health, performance and fertility of other livestock (especially poultry).

Objective 2: Phytogenic additives

We are considering the extraction and use of active substances such as oregano, thyme, wormwood, sage, cumin and their incorporation into feed mixtures for poultry, rabbits (or rats or calves) in order to reduce the occurrence of the genus Eimeria. The aim is to determine the effect of selected active substances (or their combinations) on the microbial population of the gastrointestinal tract (PCR method), the number of oocysts and possibly the effect on morphology and histology of the gastrointestinal tract and antioxidant and cytochrome P450 activity. The sensory quality of the meat will also be monitored.

Objective 3: Physical structure of compound feeds

Influence of different structure of feed mixtures (in non-pelleted and pelleted form) on morphometry and histology of sections of the digestive tract of poultry and calves. The aim is to find out how particles of different sizes in the feed mixture affect nutrient retention, digestive viscosity, health and metabolism. The structure of faeces / faeces will also be monitored and compared with the structure of the feed mixture.

Objective 4: Insect protein

To obtain more general knowledge, it is possible to perform an experiment on rats with the addition of insect protein in the feed, where it is possible to analyze the viscosity of digestion, morphometry, histology, digestibility of insect protein. Our workplace deals with the use of insect products in poultry nutrition. The aim is to introduce and develop a method for determining the in vitro digestibility of nutrients in dog food. The aim is to compare feeds with insect protein content and without insect protein content.

Objective 5: Probiotic cultures

As this objective is very complex, it is composed from several subtasks - to complete the development of a probiotic mixture for poultry; to complete the development of a probiotic mixture for newborn piglets; to prepare a probiotic mixture for weaned piglets; to define the properties of bacteria of the intestinal microbiota that are associated with the mucoid layer of the chick intestinal epithelium; to identify feed supplements that will promote the multiplication of desirable bacteria from probiotic mixtures for chicks and to prepare a microbial mixture for modification of the environmental microbiota.

The main volume of work in the activity leading to complete the development of a probiotic mixture for poultry will be directed towards the definition of the final microbial composition of the preparation. Not only must the product be safe and effective for chicks, but it must also be easy to prepare and distribute. While the composition for maximum safety and effectiveness is almost known, it will be necessary to develop the simplest possible production method. Currently, we perform fermentation of 30 bacterial isolates simultaneously, but we know that after culture we detect between 10 and 15 isolates in an acceptable proportion in the final fermented product, depending on the culture medium used. An alternative approach, which we have started to test, is to divide the bacteria into multiple groups according to their growth rates and fermentation, e.g. in three separate bioreactors. The first mixture will contain fast-growing bacteria, the next will consist of medium-growing bacteria and the third fermentation mixture will contain only slow-growing bacteria. By the subsequent combination of all fermentation mixtures we will obtain a product with approximately the same proportion of all bacteria. Even though such a task may seem simple, it requires long and repeated testing. Relocation, for example, of just one bacterium from the medium- to the slow- growing group will affect the growth ratios of the bacteria remaining in the medium-growing mixture, as well as the slow-growing group ratios after enrichment with the new bacterium. Even so, we are able to resolve this activity in the first three projects.

The next essential activity will be to convert the product from its current frozen form into the lyophilised form. In addition, a limited number of lyophilisation experiments performed by external subjects have shown a considerable sensitivity of gut microbiota bacteria to lyophilisation process. However, there should be a solution, because there are two commercial products in the lyophilised form that are similar in composition. At the same time, however, we know that the stability (in fact, instability) of lyophilised bacteria in these commercial products is one of the reasons why these products are not widely used by poultry farmers. By the end of the project we should reach a level of lyophilisation suitable for commercial use.

Poultry health can be improved not only by administering probiotics but also by adjusting the composition of feed mixtures. These can either further support the probiotics administered or be used in poultry production regardless of the probiotics. In the previous period, we tested new feed supplements based on organic acids together with plant extracts. These products are prepared by Addicoo s.r.o. and are used to extend the range of their feed products. Innovative feed formulations are and will be tested for positive correlations with production parameters such as nutrient conversion or weight gain, as well as for their relationship to increasing the resistance of chicks to Salmonella and Campylobacter infection. New product launches can be expected in the first three years and in the second half of the project, with approximately three new products in each time period.

In parallel, the activity "to complete the development of a probiotic mixture for newborn piglets" will consist in verifying whether 4 bacteria that are part of a probiotic preparation for newborn piglets can be fermented simultaneously. Even if co-fermentation fails, separate fermentation of each of the 4 cultures is acceptable for industrial production. Therefore, experiments with lyophilisation will be more important. However, unlike bacterial species for poultry, we know that isolates from pigs tolerate lyophilisation much better. Therefore, it is reasonable to expect that in the third year of the project we will be able to offer a lyophilised probiotic preparation for newborn piglets.

Objective 6: IgY technology

IgY are bird, egg-derived immunoglobulins with man biological functions similar to mammalian IgG. Preparations based on IgY enriched vehiculum will be prepared against particular infections. In case of intestinal infections (clostridial infections, Escherichia coli, rotaviruses), they will be applied as feed supplement. In case of superficial skin infections (staphylococcal dermatitis), IgY will be incorporated into ointment base. IgY will be obtained from eggs delivered by vaccinated hens.

3.4. Biotechnology in reproduction

Objective 1: Optimisation of procedures for obtaining oocytes from animals, intrafollicular oocyte transfer (IFOT)

Within this objective, we intend to focus on optimising the conditions for obtaining germ cells from ovaries of stimulated animals. Since the introduction of intravital oocyte recovery by transvaginal ultrasound oocyte aspiration, it has been possible to repeatedly obtain oocytes from the highest quality animals. This technique, also known as ovum pick up (OPU), continues to evolve rapidly. The number of embryos produced by the OPU-IVP method now far exceeds the number of embryos obtained after repeated stimulation and embryo flushing (out of the global production of about 1.1 million embryos in 2019, IVP embryos accounted for about 2/3). This shows that, nowadays, OPU-IVP is a reliable and affordable technique commonly used in cattle reproduction, although the most suitable system of donor preparation is still being sought. The systems of OPU organization in donors are different, OPU can be performed repeatedly at intervals of 3-4 days or 1 week in cycling animals or even in pregnant animals. In order to increase the number of oocytes obtained from a single aspiration and improve their meiotic competence, follicle growth can be stimulated with follicle-stimulating hormone (FSH). Various protocols for hormonal stimulation of sexually mature oocyte donors prior to OPU have been described. The method is based on repeated administration of FSH, with 6-8 injections 12 hours apart, followed by a period of up to 48 hours of *in vivo* oocyte maturation, and then OPU is performed. In an effort to reduce the laboriousness of the protocols and improve donor well-being, a method to reduce the number of FSH injections is being sought. The use of substances causing a slower release of the administered FSH so that the number of stimulations can be reduced to 1-2 injections is also important. In addition to a significant reduction in laboriousness of the stimulation process, it is evident that the welfare of oocyte donors is improving. Therefore, in our project we intend to focus on optimising the stimulation frequency and we plan to test suitable carriers of recombinant FSH.

Currently, oocyte recovery from prepubertal heifers followed by IVP is a fixed part of the ART system. The OPU method of intravital oocyte recovery in heifers is possible as early as 6 months old, but is more effective in about 10 months old heifers. The laparoscopic ovum pick-up (LOPU) approach is used for oocyte recovery in the youngest age categories of large ruminants. Compared to sexually mature donors, oocytes obtained from prepubertal animals show some differences, resulting in reduced meiotic competence and lower IVP embryo production. Nevertheless, this donor category provides high oocyte gains, and so the total production of blastocysts suitable for transfer is sufficient for practice and is close to that of sexually mature donors. The use of prepubertal heifers is therefore the subject of intensive research, with a focus on maximising the yield of oocytes from a single recovery.

In vitro culture of bovine embryos is nowadays massively used on a global scale. It should be noted that it induces a number of changes in embryos, including epigenetic modifications, and the efficiency of *in vitro* embryo production in cattle is still around 20-40% of blastocysts produced from oocytes included in the IVP process. Alternatively, a technique of oocyte transfer into the preovulatory follicle has been developed to avoid long-term *in vitro* culture. The method is based on transfer of donor oocytes into preovulatory follicles of recipient females that are subsequently inseminated. As a result, oocyte maturation, ovulation, fertilisation and early embryo development take place in the natural environment of the follicle, fallopian tube and uterus of the recipient. Seven-day-old embryos are collected via routine lavage of the uterine horn or by another method (endoscopic lavage of the fallopian tube, collection from an isolated uterus after slaughter of the animal). If GV oocytes are used, they remain outside the physiological environment for a very short time during this procedure, because they are transferred into the follicle of the recipient animal almost immediately after collection from the donors (from ovaries of slaughtered animals or from live animals). The time when the oocytes are outside the female's body is in the order of hours, which is essentially negligible compared to conventional IVF, in which the whole process takes 8 days. However, the method is very complex and does not yet provide good and consistent results. In our project we intend to focus on some technical aspects of this method and improve the results obtained. This method could provide an alternative to *in vitro* embryo production.

Objective 2: Optimisation of *in vitro* culture and micromanipulation of oocytes and embryos

The method of *in vitro* embryo production in cattle has been developing since the 1970s and all important steps of the whole technology have been gradually mastered, including oocyte maturation, sperm capacitation, oocyte fertilisation and embryo culture. The success rate of this method ranges between 20-40%, reflecting different workplaces and culture systems, and is comparable to results in human ART. Our aim will be to push the success rate of embryo development to a higher level, towards 50%, so that the genetically valuable material obtained by aspiration from preovulatory follicles can be optimally utilised. We intend to focus on a few possibilities that have recently shown promise in the global use of ART in farm animals and especially in cattle. Firstly, it is possible to influence the chemical composition of the medium in which culture and fertilisation take place. For example, replacement of foetal serum in the culture medium has been shown to increase blastocyst production. Concurrently, the addition of certain specific amino acids has led to the same result; for example, an increase in proline levels during fertilisation can improve the

development into blastocysts. The combination of growth factors in the medium, such as FGF, EGF, etc., has a similar effect. Therefore, we will look for possibilities to significantly influence the number of embryos that develop into blastocysts suitable for transfer by manipulating the chemical composition of the environment for embryo development. Next, we will focus on mechanical properties of the culture medium. It has been shown that the physical and chemical properties of culture surfaces can be a limiting factor for proper cell development under *in vitro* conditions. By modifying culture surfaces and creating three-dimensional (3D) carriers that are more similar to the environment in the body, significant improvements in the properties of cells, tissue and organ cultures have been achieved. Three-dimensional carriers have been shown to improve the developmental competence of embryos, including primate and bovine embryos, as well as the success rate of embryo development after transfer. The main objective of this part of the project will be to introduce 3D culture systems suitable for the culture of bovine embryos based on a bioresorbable and biomimetic hydrogel. Our preliminary results showed, in agreement with the results achieved in laboratories around the world, that the selected materials have a positive effect on embryo development during a short-term culture. We intend to test the possibilities of long-term culture with the use of these materials and the survival rate of embryos produced in this way after transfer. We assume that the designed system will increase the developmental competence of embryos and, consequently, the percentage of blastocysts suitable for transfer. We also intend to test the effect on embryo growth using biomimetic approaches, in which bioactive substances with a stimulatory effect (growth factors, hormones, ECM proteins, etc.) will be present in 3D culture materials. This procedure has been shown to be more natural for the cells and improve their growth properties compared to growth factors dissolved in the medium.

In our project we also intend to start to use the intracytoplasmic sperm injection (ICSI) method in cattle. This method is commonly used in human ART, where its use prevails over *in vitro* fertilisation. Its use has been mixed success in cattle, mainly due to the fact that insufficient attention has been paid to its optimisation, because *in vitro* fertilisation is relatively successful in cattle, unlike in humans. However, recently, its use in bovine reproduction has received more attention due to the possibility of using immature sperm obtained from genetically elite animals before entering reproduction. We consider this method as crucial for the optimal use of germ cells from genetically valuable animals of both sexes. Firstly, it is possible to obtain the developmental stages of sperm suitable for fertilisation from bulls before reaching sexual maturity and, secondly, the success rate of fertilisation using ICSI in aspirated oocytes from genetically valuable heifers will be higher than using conventional *in vitro* fertilisation. The introduction of the method to our workplace is made possible due to the technological equipment of the workplace and the experience of the staff with these techniques performed in model animals.

Objective 3: Evaluation of *in vivo* and *in vitro* obtained germ cells and embryos

Within this objective, we plan to evaluate the quality and development of germ cells and embryos. Our goal will be to develop methods that have a strong evaluative value and that can be used for the assessment of the properties of germ cells and embryos under operational conditions. These methods will also allow us to evaluate germ cells and embryos produced within the other objectives. Regarding embryos, we intend to focus on two procedures - invasive methods based on the collection of cellular material from embryos, which

will allow accurate assessment of the quality of the embryos or oocytes influenced by, for example, different culture methods. Next, we intend to focus on non-invasive methods, namely the analysis of extracellular vesicles, which can provide crucial information on embryo quality without affecting embryo development. Regarding invasive procedures based on obtaining the cellular material from embryos by micromanipulation, this material will be further characterized using a set of genes for screening we have developed for this purpose. The expression levels of these genes will be measured using quantitative polymerase chain reaction (qPCR). The genes with the highest expression level during early development in the blastocyst stage were purposely selected. This will allow us to objectively assess whether full embryonic development has occurred and thus assess, for example, whether the culture medium modification is appropriate or not. Non-invasive methods of development assessment have recently been on the rise. Two procedures are most commonly used in human ART. The first is monitoring of the development using time-lapse microscopy. This procedure allows the analysis of morphokinetic properties of embryos during early *in vitro* development and allows the improvement of transfer success rate by selecting suitable embryos based on their developmental dynamics. This method is also used in our laboratory and we intend to use it in this project to evaluate the *in vitro* produced embryos using ICSI. Another very promising method is the analysis of extracellular vesicles. These are membrane-coated vesicles which are released by cells into the external environment and the main function of which is cell-to-cell communication. It has been shown that embryonic cells also communicate with each other in this way and that these vesicles can be isolated from the culture medium. The vesicles contain a variety of substances, from proteins to lipids to nucleic acids. Their analysis is currently receiving a great deal of attention worldwide and the analysis of the content of small non-coding micro RNAs, which accurately inform about the embryo status and quality, appears to be the most promising. In our project, we intend to focus on the introduction of procedures for the isolation and analysis of these structures. The aim is to introduce a non-invasive method of selecting high quality embryos for transfer and a method of evaluating changes induced in the culture environment, such as the effect of growth factors or 3D culture.

Objective 4: Conservation of nuclear material of oocytes and embryos

Not all oocytes and embryos obtained from them are of adequate quality to ensure full development. High-quality nuclear DNA (nucleus, chromosomes) can thus be localized in the cytoplasm, which, on the other hand, is of very poor quality. The aim of this part of the project is to develop procedures that would ensure long-term preservation of nuclear material without losing its viability - germ sac, chromosomes of maturing and mature oocytes, primordial nuclei so that after thawing this material can be used to reconstruct appropriate cells with quality developmental competence.

Currently, the procedures are considered for human assisted reproduction to eliminate mutated mitochondrial DNA. Their use is also common in laboratory animals. These methods have not yet been used in cattle. Their importance would be evident especially in genetically valuable individuals, where there are problems with poor quality cytoplasm and then in the classical breeds of cattle, which fall into the category "Genetic animal resources".

In our project, we also intend to focus on semen analysis and sperm selection for artificial insemination of cattle and pigs. Sperm analysis using more accurate and objective semen quality parameters will improve the fertility of sires in artificial insemination. Artificial insemination technology for cattle is generally more advanced than for other farm animal species. It mainly uses frozen sperm, which is suitable for its transport and storage. Unlike cattle, insemination in pigs is carried out using diluted chilled, not frozen, insemination doses. Extenders and preservation media have seen a great deal of innovation over the years, but the durability of diluted semen is an area that continues to receive attention. Light microscopic evaluation of sperm provides useful information about a given semen sample, but due to its subjective nature has a limited prognostic value. Cryptic sperm abnormalities occurring at the molecular level are not easily detectable by light microscopy, but can be detected by a number of biomarkers. This modern approach is referred to as biomarker-based sperm analysis. This includes fluorescent markers of the acrosome status, fluorochromes detecting altered chromatin integrity or sperm DNA, vital dyes detecting sperm mitochondrial activity, probes detecting apoptotic events, and antibodies detecting proteins that are increased or decreased in defective sperm. The most suitable method of testing of a number of the above biomarkers is flow cytometry, which allows rapid, automated, high-throughput and objective measurement of the relative amounts of these biomarkers in sperm. The potential for biomarker-based analysis is high, as new biomarkers of sperm quality continue to be identified. This technology can be further improved to sort sperm based on different levels of fluorescence emitted by individual spermatozoa, which is how sperm is currently sex sorted based on the quantification of fluorescence emitted by DNA staining. Computer-assisted sperm analysis (CASA) also evaluates various sperm quality markers more accurately than conventional light microscopy and has been extended to analyse sperm viability and DNA fragmentation. At present, CASA is a simpler and more affordable option that allows rapid and repeatable assessment of sperm motility and morphometry. Another rapidly developing method of sperm selection is nanopurification, which uses various negative biomarkers to remove abnormal or defective sperm. Magnetic nanoparticles are coated with probes such as lectins, recombinant proteins and antibodies known to bind negative biomarkers found on the surface of abnormal sperm. The bound spermatozoa can then be removed from the sample using a magnet without the need to damage the sperm by centrifugation or filtration. Regarding sperm storage, extenders are important. The use of chilled insemination doses is the preferred method of assisted reproduction in all farm animal species except cattle. Boar spermatozoa in particular are highly sensitive to severe cold shock, and therefore sperm that undergo freezing are often weakened. In the weakened sperm, DNA fragmentation, degradation of proteins and RNA present in sperm, disruption of the acrosome and sperm membranes, as well as reduced mitochondrial activity and sperm motility can occur. All these problems can lead to reduced fertility, and therefore boar sperm extenders are still the subject of extensive research to improve storage efficiency. The aim is to improve semen analysis using more accurate and objective semen quality parameters, including particularly the evaluation of the quality of cooled and frozen insemination doses in all farm animal species. The development of computer-assisted semen analysis (CASA) allows a more detailed analysis of different characteristics of sperm movement, accurate concentration and detailed morphological analysis of sperm defects. By using the Sperm Chromatin Structure Assay (SCSA) in different livestock species we can obtain the information on sperm DNA quality, which can be affected by genetics, hygiene of the environment and animal welfare. Furthermore, the development of flow cytometry and nanopurification methods for the

evaluation and purification of insemination doses is necessary as well as the development of methods for preservation of frozen genetic material, especially boar sperm, but also sperm from various rare species of the Bovidae family. It is necessary to introduce the following methods into practice: the HOS test to improve boar semen extenders and to test animals suitable for the production of frozen insemination doses; the PSA assay to evaluate acrosome integrity by pisum sativum agglutinin binding activity (FITC-PSA) using fluorescence microscopy; and the NBT assay to measure the mitochondrial spiral activity of sperm from individual sires.

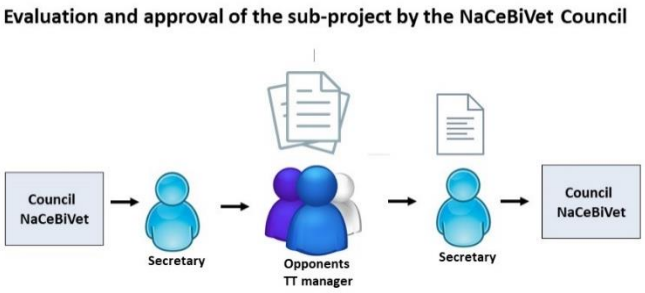
4. Description of strategic project management and principles for the composition and proceedings of the Centre Council

The Council, which is composed of representatives of the public and private sectors, plays a dominant role in the organizational structure of the Center. Its main task is to determine the strategic direction of the center and to implement fundamental decisions concerning its operation. The Council decides on the content and conditions of the internal call for sub-projects, discusses and approves requests for changes to the Centre's project concerning results, objectives, professional agenda and other binding parameters of contracts, discusses and approves sub-projects and discusses interim and final reports of sub-projects and decides on measures to eliminate deficiencies. The center has its own project manager, who acts as the coordinator ensuring the administration and management of the center, and as a contact person for internal and external communication. The manager is responsible for the operation of the center, coordination of project topics, fulfillment of the center's goals, and also mediates communication between members and towards external institutions. The manager's competencies also include supervising the fulfillment of the work schedule according to the Parameters of the project solution and managing changes in the project, both in terms of administration and change management according to the project rules and Provider's methodological instructions. He is responsible to manage the administrative team, which consists of a financial manager and an administrative worker and works closely with the technology transfer manager. The activities related to IPR protection and commercialization are coordinated by the technology transfer manager in cooperation with the Council members and with the solvers of individual sub-projects. The Secretary of the Council is responsible for administrative and organizational requirements related to the activities of the Council and for the preparation, administration of minutes and sound recordings of the meetings.

The Council of the Center is the main decision-making body that manages the implementation of the Center. It determines the conceptual and strategic direction and supervises the fulfillment of the strategic research agenda. It is composed of representatives of public administration, research, application and an adequate part of the members is from abroad. The NaCeBiVet board has 8 members (4 internal and 4 external). Its chairman is the principal investigator of the project, who is employed by the main beneficiary.

Evaluation and approval process of the subproject

The submitted subproject proposal must be forwarded to the evaluation and approval process, including the NaCeBiVet Council and the TA CR. The Secretary of the Council ensures the evaluation of the subproject and prepares the final report. For the purposes of the evaluation, all members of the Council are addressed, with exclusion of members who could be in conflict of interest. These members in role of opponents complete a report including a statement on the set criteria and verbal evaluation. One of the opponents is the manager for technology transfer and intellectual property protection, the employee as the main beneficiary of the support, who comments on the criteria verbally on the entire budget. On the basis of the reports, the Secretary prepares a final report, on the basis of which the Council decides on the support of the project. After the approval of the proposal by the Council, it is forwarded to the TA CR together with the final report and the anonymized version of the opponents' opinions in accordance with Program conditions.



5. Method for evaluating the implementation of the strategic agenda - evaluation plan

The project is scheduled to begin in January 2023 and finish in December 2028. Part of the funding requirement is the undertaking of the evaluation to:

monitor project processes and analyse critical success factors and factors that impeded success;

assess the achievement and potential scalability and sustainability of project outcomes.

This chapter evaluates the specific activities by which the general principles and mechanisms of operation described in the project application were fulfilled in individual years.

Networking, relationships and cooperation

During the period under review, it will be evaluated whether care has been taken to interconnect member institutions within the consortium and to strengthen relations with external partners. For different purposes and specifics of both categories, internal and external relations will be evaluated.

Cooperation within the consortium

Relationships within the consortium enable fruitful cooperation on sub-projects and beyond, as well as effective sharing of infrastructure and experience. The building and deepening of internal relations will be realised within NaCeBiVet, usually in four phases:

Regular presentation of the possibilities and needs of individual entities at meetings of the Council of the Center or at workshops,

Dedicated meetings, where the method of future cooperation and its outputs are formulated on the basis of thematic and interest interplay.

Consolidation of relations in the form of cooperation on a sub-project, another grant or contract project.

Ensuring the most effective possible cooperation by staffing the involved institutions.

As part of the evaluation process, the fulfilment of individual phases of cooperation and the results from them will be evaluated.

Cooperation with external partners

In the monitored period, the intensity of this cooperation will be monitored and evaluated, and the results of successes with external partners. Submitted projects of member and non-member institutions in other programs of TA CR and MEYS or other providers will be monitored, as well as the intensity of establishing contacts with other entities as potential implementers of innovation outputs.

The Centre's development strategy will be drawn up and submitted to the Council for approval.

Sub-projects

The advantage of subprojects is the fast and operative launch. Thanks to the systematic work of the Council, the evaluators and the appropriate budgetary conditions, it is possible to respond to industrial demand in a matter of weeks, which can mean a decisive competitive advantage. Within the monitoring period, the operability of elaboration of sub-projects and other aspects will be monitored, such as the involvement of young researchers in the development and application of new biotechnologies.

Scientific direction of the Center -The body determining the overall direction of the Center is the Council of the Center.

Financial management and commercialization - evaluation of continuous drawing of the subsidy

Education – conferences - presentation of the latest scientific knowledge in the field

Popularization - introducing new knowledge and biotechnologies to the general public

Website - one of the goals of the Centre's new website is to raise awareness and education in the field of new biotechnologies in veterinary medicine.