OTKA SNN 116993 – Scientific report

Initial goals of the project and main factors influencing the implementation

This Hungarian-Slovenian bilateral research project has started in September 2015. In the preparatory phase (i.e. during the project planning and proposal writing) the participants have been intensively reviewing the available scientific literature and were discussing with experts in the field (particularly Professor Silvia Cappellozza, head of the Sericulture Unit of the Council for Agricultural Research and Agricultural Economy Analysis - Consiglio per la Ricerca e Sperimentazione in Agricoltura, Unità di Ricerca di Apicoltura e Bachicoltura (CRA-API), Padova, Italy). It was necessary, because sericulturing was ceased in the 1960's both in Hungary and in Slovenia, therefore no current information was available on the problems, challenges and feasibility of this activity in the two countries. The research hypotheses and aims were based on experiences and reports from Asian countries (China, India, Japan), Italy and Bulgaria, where uninterrupted sericulture was practiced for hundreds or thousands of years.

The studies within this project had dual aims as: i.) answering fundamental research questions on the diversity and nutritive characteristics of mulberry trees native in Hungary and Slovenia, identification and genetic characterisation of silkworm pathogens, and description of components of the silkworm immune system, and ii.) assessing the feasibility of sericulture in the XIXth century in the two countries, providing information on the optimal feeding sources (i.e. mulberry sorts), identifying risks (e.g. infectious diseases, environmental conditions, technology) that might influence the success of sericulture or the quality of the end product (i.e. raw silk). Hence, the research project provided both novel scientific results (partially published, predominantly under publications) and practical recommendations to serve as a technical basis and support for the reintroduction of sericulturing in these countries.

During the implementation of the research, we have experienced several, unexpected difficulties and obstacles, and the results of the studies in the first years also forced us reviewing and reconsidering our hypotheses and modify the aims of the studies accordingly. This flexibility allowed us to use the resources (the grant support) effectively. All changes were communicated in the annual interim reports, were requested at, and approved by the NKFI office. The most important changes in the research, compared to the initial research plan and timing are the followings:

- The main comparative feeding experiments were planned for the third year of the project, using local (i.e Hungarian and Slovenian) mulberry sorts and "commercial" ones selected and used for sericulturing in Italy. Mulberry seedlings from an Italian nursery were purchased and planted in Hungary and in Slovenia in the autumn of 2015. Unfortunately, the repeated, late, strong freezes in the spring of 2016 caused significant losses in the plantations (>50%), which was further complicated by the dry summer months. New plants were planted in the autumn of 2016 (and later), but losses (due to spring freezes and summer draught) were experienced in the subsequent years. Therefore, the growth of the plants was slower than expected and originally planned. Consequently, we had to postpone the main (controlled) feeding experiment to the last spring/summer of the project. This generated delays in obtaining results and publishing them in scientific journals.
- In the first years of the project, silkworms were reared both by the Hungarian and Slovenia partners, as well as by volunteers (without particular previous experience) in both countries. Bacterial diseases (Flacherie) have not been observed in any of the sericultures and no pathogen bacteria were detected in the silkworms which died during rearing. However, in several cases, considerable losses were observed in the last instars. Our microbiological studies revealed that the *Bombyx mori nucleopolyhedrovirus* (BmNPV) was the causative agent of the disease (Grassserie) outbreaks. Therefore, the focus of the microbiological studies was turned from bacteria towards viruses.
- The advances in technology (and reduction is service costs) allowed us to include metagenomic and gut microbiota studies using next generation sequencing methods.
- The Principal Investigator of the project was on maternity leave between 06/2016 and 05/2017. Due to the nature of the experiments (seasonality, as green mulberry leaves are only available between May and September), the project was interrupted for 12 months, with the approval of the NKFI Office.
- In the spring of 2020, the lockdown measures and international travel restrictions connected to the COVID-19 pandemic made the implementation of the main comparative feeding experiment impossible. Therefore, we have requested and were approved for one year extension of the study period (without changes in the total amount of the grant support).

In this final scientific report, we would like to provide point-by-point reflections and answers to the hypotheses and key questions of the original (2015) research plan (quoted *in Italics*), and describe the activities and results obtained within the research. However, we have to emphasize that – due to the above-mentioned delays in the implementation of the main experiments – the majority of the results has not been published in scientific journals yet. Hence,

according to the rules of NKFI Office, we would like to ask that publications connected to this project in the forthcoming one year should be taken into account in the evaluation of the success and impact of the project.

"Hypotheses, key questions, aims of the project
Hypothesis:
1) The bacterial pathogens involved in the Flacherie disease of the silkworm are not fully characterised. It is likely that mycoplasma species also play a role in the disease complex."

- Flacherie disease was not observed in any silkworm rearing settings in Hungary and Slovenia and no pathogen bacteria (including mycoplasmas) were detected in the ill or dead larvae. Therefore, studies on the role of bacteria in the pathogenesis of Flacherie disease was not possible to investigate.

"2) Neuraminidase is an important factor of pathogenicity of bacteria involved in Flacherie disease."

- No Flacherie disease was observed. Neuraminidase activity was not detected in bacteria isolated from ill or dead silkworm larvae.

"3) Mulberry leaf quality plays a significant role in development, silk production, and resistance of silkworm against bacterial pathogens. Our hypothesis is that feeding silkmoth hybrid larvae with leaves of certain, old, local Hungarian and Slovenian mulberry genotypes has a positive effect on the development, immune reactivity, general health status of larvae, and production of silk."

- The effect of 16 Hungarian, 10 Slovenian and 4 "commercial" mulberry genotypes were investigated in controlled feeding experiments. The investigated parameters (growth and losses during rearing, immune reactivity, health status of larvae, and cocoon and silk rope quality) were similar in the different experimental groups. However, statistically significant differences in certain parameters were detected. The final results indicate that the silkmoth hybrid larvae currently used in sericultures in Italy can produce high quality cocoons if being fed with leaves of certain, local Hungarian and Slovenian mulberry genotypes.

"4) The information on the number of remaining old mulberry varieties in Hungary and Slovenia and the taxonomic classification at the species and subspecies level is unreliable and incomplete"

- Within the project, old mulberries (> 100 years of age / > 180 cm in trunk perimeter) were identified and catalogued in Hungary (1498 locations, out of that 24 tree lines) and in Slovenia (645 trees). Samples from the trees were collected and grafted. Genbanks including 255 Hungarian and 133 Slovenian genotypes were established. Taxonomic classification revealed the predominance of *Morus alba* L. Genetic comparisons of genotypes using 6 SSR markers were performed.

"5) The old varieties are valuable genetic resources best adapted to specific climate conditions with significant qualitative and quantitative composition of important metabolites."

- The metabolite contents of the leaves of 238 Hungarian and 643 Slovenian genotypes were analysed. The trees were analysed according to FAO mulberry descriptors and phenologically evaluated. Improved survival of local genotypes were observed, compared to the "commercial" and exotic genotypes (imported from Italy).

"Key questions:
1) Flacherie is a multi-factorial disease of silkworm. Which bacteria can be detected in the tissues of clinically healthy and Flacherie disease affected silkworms in Europe?"

- Flacherie disease was not observed in any silkworm rearing settings.

"2) Neuraminidases play significant role in the pathogenesis of several bacterial diseases of vertebrates. Do the bacteria involved in the Flacherie disease of the silkworm have neuraminidase activity, and does it have impact on the development of clinical signs and pathological lesions?"

- Neuraminidase activity was not detected in bacteria isolated from ill or dead silkworm larvae.

"3) Primary and secondary metabolites in the mulberry leaf have significant effect on the health of silkmoth larvae. Do leaves of local mulberry genotypes have sufficient concentration of sugars, proteins and amino acids necessary for growth, development of larvae, disease resistance and quality silk rope production?"

- Based on multivariate analysis comprising the leaf nutritional contents of selected Hungarian and Slovenian mulberry genotypes and silkworm growth parameters we found differences compared to the reference cultivars selected for silkworm feeding. The group of Hungarian genotypes correlated positively with total

proteins, total phenolics and certain individual phenolics, which further correlated positively with silkworm weight. However, there was no significant difference between the cocoon quality of larvae fed with selected Hungarian and Slovenian mulberry genotypes and reference varieties.

"4) Many phenols in plants can serve as antinutritients to phytophagous insects at relatively low concentrations. Phenolics reduce the nutrient value of tissues by binding amino acids and proteins and they can even reduce digestion of insects by binding to digestive gut enzymes. Do leaves of local mulberry genotypes have high concentration of phenolics and a negative impact on growth and development of larvae?

- Negative effect on the growth and development of larvae fed with selected Hungarian and Slovenian mulberry genotypes was not observed. In the silkworm feeding experiment we were able to determine a strong correlation between predominant phenolics (chlorogenic acid, rutin, kaempferol acethylhexoside and isoquercetin) and the weight of 5th C/5-7th D silkworms. All selected HU and SI genotypes gave cocoons of the highest quality when compared to reference sericultural varieties. The multivariate analysis enabled us to select superior mulberry genotypes in respect to silkworm weight and cocoon yield.

5) Low molecular weight thiols (cysteine, glutathione) and ascorbate in mulberry leaves may have significance in nutrition and health status of silkworms. Do mulberry leaves of selected genotypes with higher concentration of glutathione have any significant effect on development and resistance of B. mori to bacterial diseases?

- As bacterial diseases were not diagnosed in any sericultures, this question could not have been answered by the current research.

The original aims of the project:

1: Qualitative and quantitative analysis of bacterial infections and loads in clinically healthy and diseased silkworm. Special emphasize will be put on detection of mycoplasmas.
2: Determination of neuraminidase activity of pathogenic bacteria isolated from B. mori.
3: Development of a model system using B. mori and their specific bacteria to investigate the effects of feed on bacterial multiplication, development of clinical signs and expression of immunity related genes.
4: Collection data regarding locations of mulberry plants and creation of digital map and digital library.
5: Propagation and conservation of old local mulberry genotypes from different eco-geographic regions of Hungary and Slovenia, which coincided with the sericulture industry in the past.
6: Molecular analyses (AFLP, SSR) to identify genetic variation of available local Hungarian and Slovenian mulberry genotypes.
7: Qualitative and quantitative screening of important primary (sugars, proteins and amino acids) and secondary metabolites (phenolics, ascorbate, glutathione) in leaves of different local mulberry varieties in different developmental stages to test their relevance on development (nutrient digestion, growth, survival rate of young larvae, cocoon weaving activity, cocoon yield and filament quality) and health status (resistance against specific pathogens, improved innate immunity) of larvae.

Description of research activities and main results of the studies:

Ad 1.: **Identification of microbes from healthy and diseased silkworms:**

Clinically healthy and diseased *Bombyx mori* larvae have been collected in silkworm breeding farms in three consecutive years (2016-2018) in Hungary, Slovenia and North Italy. In Hungary, five to seven rearers (sericulture at the University of Veterinary Medicine and volunteers in different parts of the county), in Slovenia seven to eight rearers (sericulture at the University of Maribor and volunteers in different parts of the county) and in North Italy 10-15 rearing farms were sampled within the three years. From each sericulture, at least three larval samples and *Morus alba* leaves have been collected. Haemolymph was collected from the larval samples and was inoculated on blood-agar plates. Bacteria were isolated from 30 to 60% of the samples. *Aerococcus* sp., *Enterococcus* sp., *Micrococcus* sp. and *Proteus* sp. dominated in the positive samples. However, many of the samples contained mixed flora with saprophyte species. Most importantly, diseased larvae did not show signs characteristic to bacterial infections (Flacherie disease), and there was no statistically supported connection between the presence of bacteria and certain bacterial species in the larvae and the clinical manifestations of any disease.

Selected larvae were homogenized and were subjected to nucleic acid extraction. Larval DNA samples were screened for the presence of *Mycoplasma* sp. with polymerase chain reaction (PCR) using universal primers targeting the 16S-23S rRNA ITS regions of all Mollicutes species, as well as the rpoB gene on mycoplasmas. Amplification products obtained in the reactions were subjected to direct sequencing in both directions using the Sanger's method. Sequences were identified by BLAST search. None of the sequences has shown similarity to mycoplasma sequences. The highest

nucleotide similarity values (97-83%) were observed with Enterobacteriacae (*Enterobacter*, *Salmonella*, *Citrobacter*, *Kelbsiella*, *Raouletta*, *Yersinia*, *Providentia*, *Shigella* spp.), *Rubrobacter* spp. and *Strentropomonas* spp.
Due to the unsuccessful attempts on cultivation of pathogen bacteria from the haemolymph of silkworms, and the lack of connections between the presence of bacteria and clinical status of the larvae, we have changed our research strategy. Because the previously collected sets of samples were different in more parameters (keeping conditions, temperature, humidity, origin and content of leaves, feeding frequencies), we decided to reduce the variables and increase sample sizes. In 2018 we have reared high number of silkworms (approximately 10,000 larvae) which were fed with leaves of old, Hungarian mulberry trees. In the fifth instar stage larvae were collected and ordered into three pools categorised as 1.: healthy ones with good development, 2.: larvae with retarded development (small size, moulting and spinning problems) but without disease signs, and 3.: diseased larvae (colour changes, inactivity, diarrhoea, death). The guts of ten larvae from each pool were removed and homogenized. The whole bodies of group 3 were also homogenised. The homogenates were submitted to metagenomic investigation to reveal the bacterial flora of the gut and to identify potential differences in the composition of the flora of the three groups, based on bacterial rRNA sequences. The gut microbiome significantly differed in the three sample types (**Figure 1.**). In group 1, approximately 2,600 different bacterial species were detected. In group 2 the number of detected species exceeded 13,000, while in group 3, it exceeded 7000. The relative amounts of bacteria also differed. For example, amongst Firmicutes, *Lactococcus spp*. represented 45% of the detected sequences in group 1, while in group 2 and 3, the ratio of *Enterococcus* and *Streptococcus spp*. was higher (12-19%), while *Lactococcus spp*. frequencies decreased (to 12-26%). Amongst Gammaproteobacteria, *Pseudomonas* and *Acinetobacter spp*. dominated in group 1 (30 and 17%, respectively), while in groups 2 and 3, *Pantoea spp*. were much more prevalent (15 and 36%). In group 1, the relative amount of *Pantoea spp*. sequences was 1%. In the 2021 rearing season the same sampling strategy was applied in a large-scale rearing farm in Slovenia and in North Italy. Additionally, gut contents of larvae reared during the comparative feeding experiment with leaves of *Morus nigra*, *Morus australis* and *Morus rubra* × *nigra* hybrid, as well as reared on artificial food were submitted to rRNA sequence identification. The sequence data were obtained from these samples; however, the BLAST alignments of the reads and the comparative analysis of the gut microbiome compositions of the different samples are ongoing at the time of writing the final technical report of the project. We plan to publish the results of these studies in a scientific article.

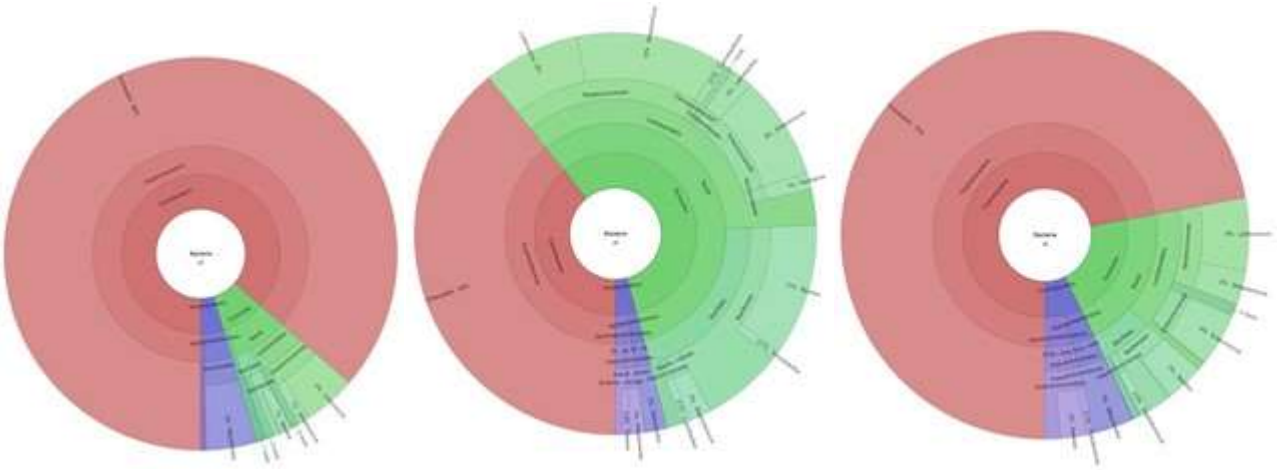

Figure 1.: Bacterial microbiome composition of the gut contents of healthy, well-developed (1), cachectic (2) and ill (3) silkworms, based on bacterial 16S rRNA gene sequences.

Certain rearers in all three countries experienced losses during in the final stage (fourth and fifth instar larvae and spinning) in 2017 and 2018. Larvae became greyish-greenish, inactive, and they died or failed to spin (**Figure 2.**). Based on the clinical signs, viral infection was suspected. Larvae were investigated for *Bombyx mori nuclear polyhedrovirus* (BmNPV, Baculoviridae) by in-house developed molecular methods (PCR, real-time PCR). The samples were found positive for virus infection. Subsequently, electronmicroscopic investigations also revealed the presence of typical occlusion bodies with aggregated, rod-shaped virions, characteristic to BmNPV (**Figure 3.**).

Figure 2.: Clinical signs of BmNPV in a fifth instar larva.

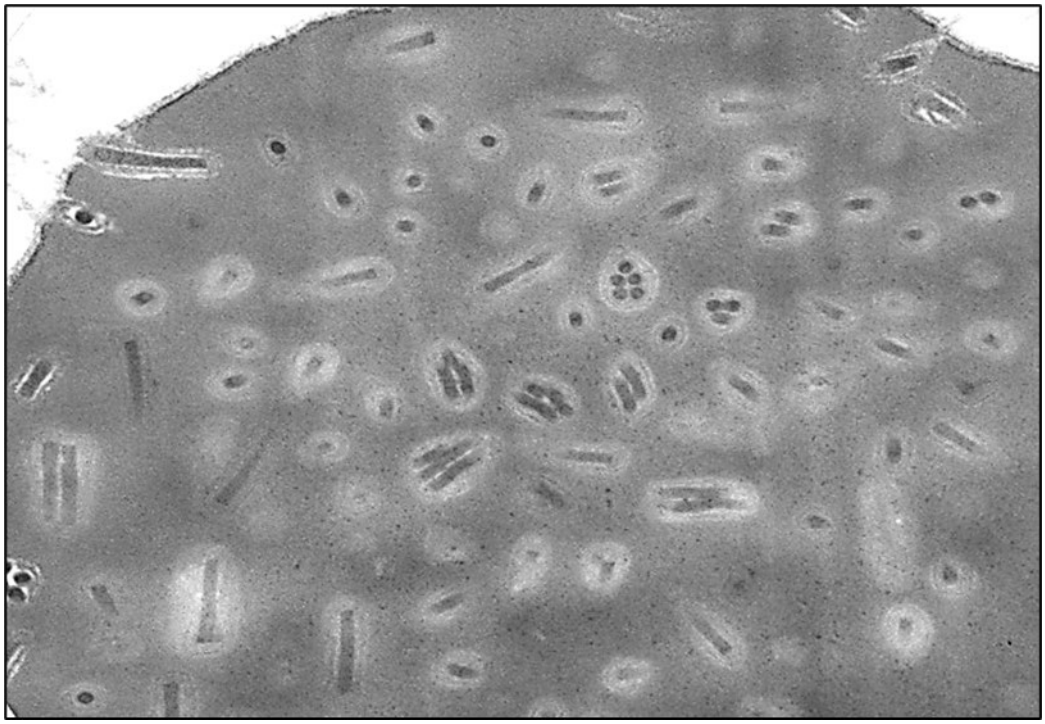

Figure 3.: Negative contrast electronmicroscopy picture of BmNPV in an occlusion body.

After literature surveys and consultation with our cooperating expert in Italy, we have recognized that in the European rearing and hygienic conditions, this virus infection poses much higher threat to sericulture than the bacterial infections. Therefore, we have initiated studies on the impact of BmNPV on silkworm rearing in Europe. Partial nucleic acid sequence determination and analysis revealed that the colonies were affected by the geno/morphotype of the virus with single nucleocapsids within the envelope of the occluded virions, and not to the ones with multiple nucleocapsids. The latter ones were described in Brazil and suspected being more virulent. BmNPV was detected in different developmental stages of the *Bombyx mori* (eggs and different larval stages) as well as in their environment (fomites) and even in ants attacking the dead larvae. Therefore, we consider that environmental contamination can play an important role in the maintenance and transmission of the virus. The viral DNA was detected in clinically healthy, five instar larvae too; however, the virus loads in ill larvae were 10^6 – 10^9 higher than in the healthy ones (**Figure 4.**). Hence, clinical manifestations, can be influenced by environmental factors, particularly temperature. For the improvement of diagnostic tools, BmNPV-specific, rapid molecular diagnostic method, based on the Loop-mediated Amplification (LAMP) technique was developed and successfully tested on silkworm samples (**Figure 5.**).

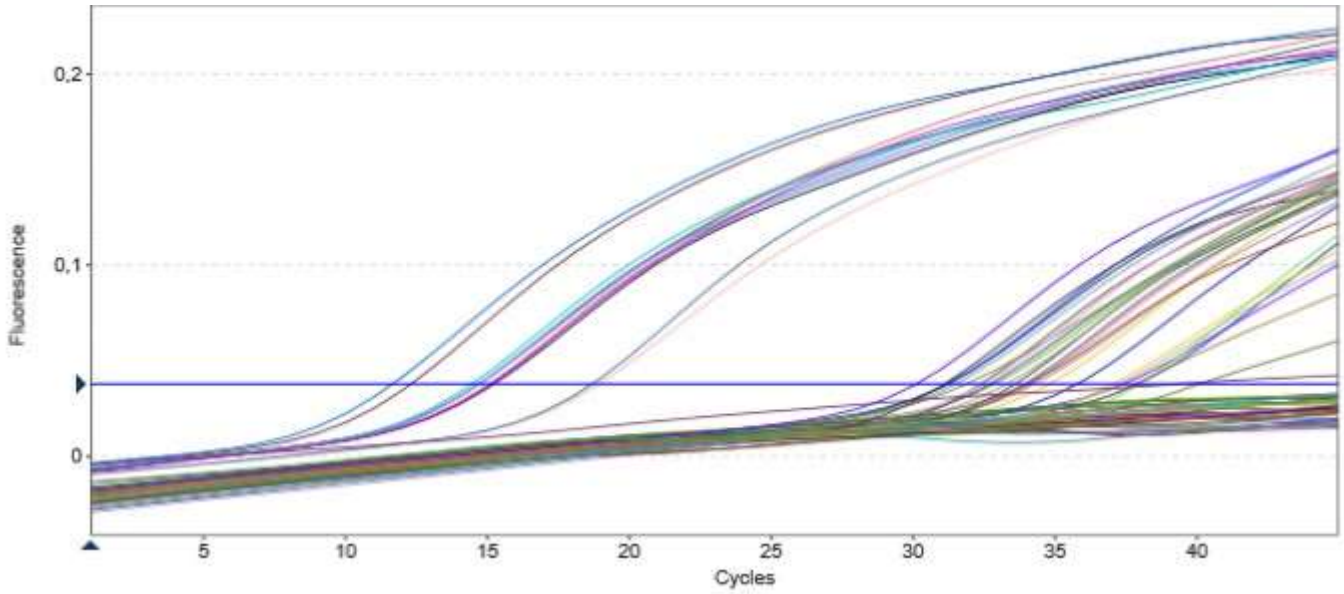

Figure 4.: Real-time PCR detection of BmNPV DNA in silkworm larvae.
Curves crossing the threshold line (horizontal blue bar) between cycles 12 and 15 are representing samples from ill larvae. Curves crossing the line after 30 cycles are from healthy larvae.

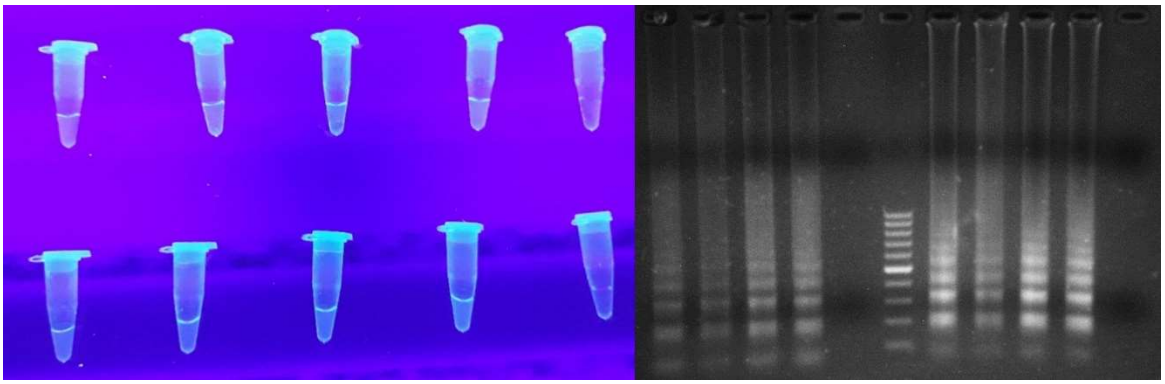

Figure 5.: Loop-mediated Amplification assay for the detection of BmNPV DNA is silkworm samples.
Left side upper panel: Reaction mixes of four positive samples and one negative sample under UV light, after 30 minutes incubation at 60ºC. Left side lower panel: Reaction mixes of the same four positive samples and one negative sample under UV light, after 60 minutes incubation at 60ºC. Right side panel: Agarose gel electrophoresis of the amplification products show on the left side panel.

In 2018, together with the gut microbiome studies (described above), the homogenates of diseased larvae (group 3) were submitted to whole genome sequencing method (using random primers – shotgun sequencing) to identify further pathogens, particularly viruses. These investigations revealed that (after exclusion of host DNA sequences) the body homogenates of ill larvae contained BmNPV sequences (73% of the detected sequences), further viral sequences similar to baculoviruses, as well as bacterial sequences (491 species) (**Figure 6.**). The nearly complete genome sequence of the BmNPV has been determined. In 2021, additional whole body samples from diseased larvae in a Slovenian and a North-Italian rearing farm were submitted for whole genome (shotgun) sequencing. The sequence data were obtained from these samples; and the comparative analysis of the data are ongoing at the time of writing the final technical report of the project. We plan to publish the results of these studies in two scientific articles (genetic characterisation of BmNPV from silkworms in Europe and development of LAMP-based diagnostic method for BmNPV detection).

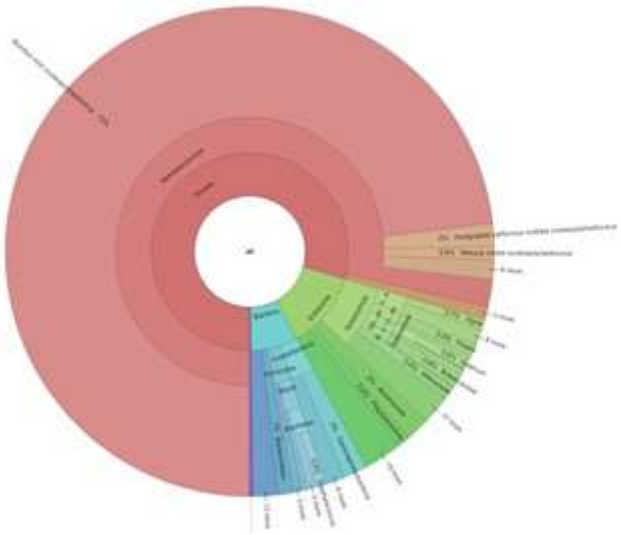

Figure 6.: Microbiome composition of the body tissues of ill larvae, based on shotgun whole genome sequencing

Previous studies of other research groups reported the presence of Red Fluorescent Protein (RFP), a chlorophyll-binding protein that is produced in the midgut of silkworms and having detectable anti-microbial, particularly anti-BmNPV effect. In cooperation with the Italian partner, we have studied the production of this protein in different silkworms lines, different feeding conditions (fresh leaves and artificial feed), and keeping conditions (light and dark). Fifth instar larvae of different silkworms lines (Nistari, which is more resistant to BmNPV infection and a Bulgarian line, which is more sensitive to it), with different feeding conditions (fresh leaves and artificial feed – which does not contain chlorophyll), and keeping conditions (light and dark) were reared. Gut homogenates were collected and RFP was extracted using previously described protocols. Partially purified RFPs were observed spectrophotometrically as well as chromatographic fractions using LC-TOF. The spectral characteristics and chromatographic profile of the isolated tetrapyrroles corresponded with methyl chlorophyllide, monovinyl pheophytin A and monovinyl chlorophyllide A. Qualitative and quantitative differences were determined between the rearing conditions and lines. Although HPLC and PAGE investigations indicated the presence of RFPs at the expected molecular size, fluorescence was not detected in the samples. The experiments have been repeated in the summer of 2021 in order to identify and eliminate potential technical mistakes. In the second experiment, remarkable differences have been observed in the fluorescence activities of gut extracts of the resistant (Nistari) and sensitive (Bulgarian) lines, as well as the gut contents of larvae fed with fresh leaves and with artificial food. The detailed analysis of chromatography and PAGE results are ongoing at the time of writing the final technical report of the project. We plan to publish the results of this study a scientific article.

Ad 2: **Investigations on the neuraminidase activity of pathogenic bacteria isolated from *B. mori*:**
Because the larvae investigated within the study years did not show signs of Flacherie disease or other bacterial disease, our hypothesis on the role of neuraminidase activity (NEAC) of bacteria as virulence factor could not be proved. Selected isolates were tested *in vitro*, but no NEAC was detected. Although sialidase activity of bacteria (e.g *Enterococcus faecalis*, *Proteus* spp) that are suspected in the aetiology of Flacherie disease have been described in the scientific literature, our studies did not reveal connection between the presence of such bacteria and the health status of the tested silkworm larvae.

Ad 3.: **Investigations on environmental effects on bacterial infections in *B. mori* model:**
As described above, no pathogen bacteria were isolated from the silkworm larvae and no bacterial diseases were observed in our studies. Therefore, in this part of the project, we were focusing on the environmental effects on the immunity of silkworm larvae. Several details of the innate immune system of insects have been discovered so far; however, there is relatively little scientific data available on the immune mechanisms in silkworms. In cooperation with the Innate Immunity Group, Institute of Genetics at the Biological Research Centre in Szeged, we have implemented studies on the haemocyte composition of silkworms, expression of certain surface markers and phagocyting activities. Haemolymph were collected from larvae fed with different mulberry leaves, morphological and quantitative analysis of the blood cells were performed. Remarkable differences in cell counts were found between the different groups of larvae (**Figure 7.**). Thereafter haemocytes were tested with labelled monoclonal antibodies developed for surface markers of *Drosophila melanogaster*. The study revealed that a subset of spherical

cells expresses a surface antigen, which is reactive to the Bp4 monoclonal antibodies raised against *D. melanogaster* granulocytes (**Figure 8.**). (The same MAb was also reactive with granulocytes of *Apis mellifera* in a previous study; therefore, it might detect a common surface marker of phagocytic cells of insects.) The phagocytosis activity of the haemocytes was tested by inoculation of larvae with inactivated, FITC-labelled *Escherichia coli*. The spherical Bp4 expressing cells (presumably granulocytes) have shown phagocytic activity. In a second experiment, the haemocytes of BmNPV resistant and sensitive *B. mori* lines and larvae fed with green leaves and artificial food (see RFP experiment above) were also analysed. The plasmatocyte/granulocyte ratio of the resistant and the sensitive lines were markedly different. A manuscript describing the findings is under construction.

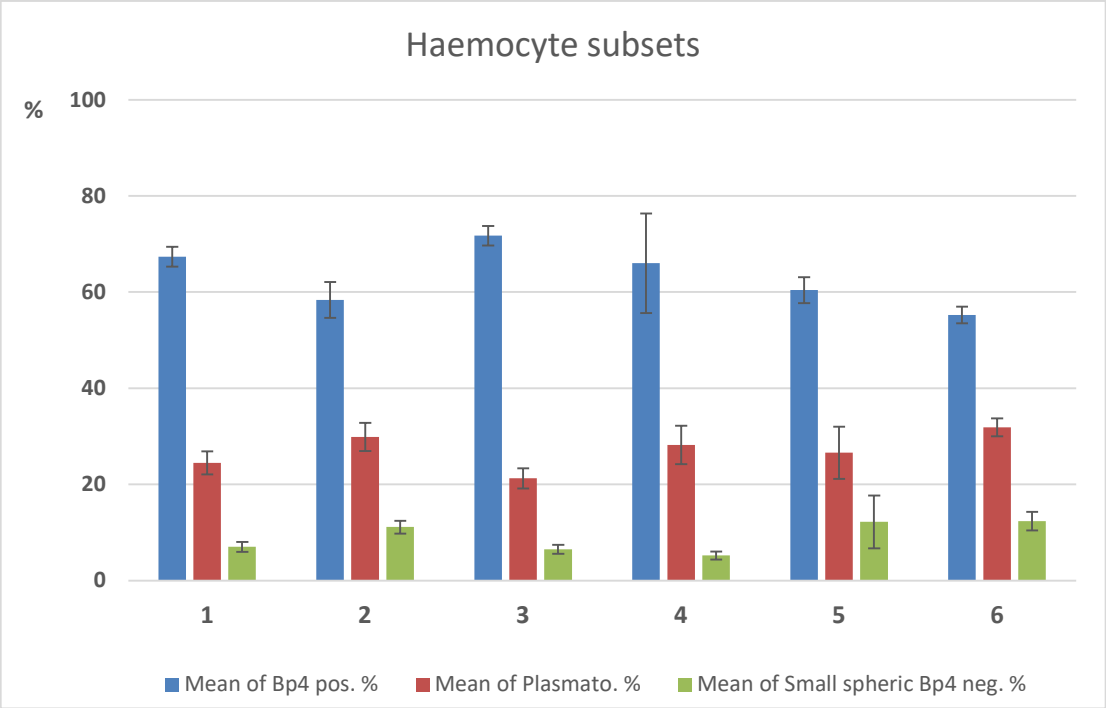

Figure 7.: Quantitative comparison of haemocyte populations of silkworm larvae fed with 1: Hungarian *Morus alba* leaves, 2: Slovenian *M. alba* leaves, 3: Italian *M. alba* leaves, 4: *M. australis* leaves, 5: *M. nigra* leaves, 6: Reduced amount of *M. alba* leaves (~50% of *ad libitum* consumption).

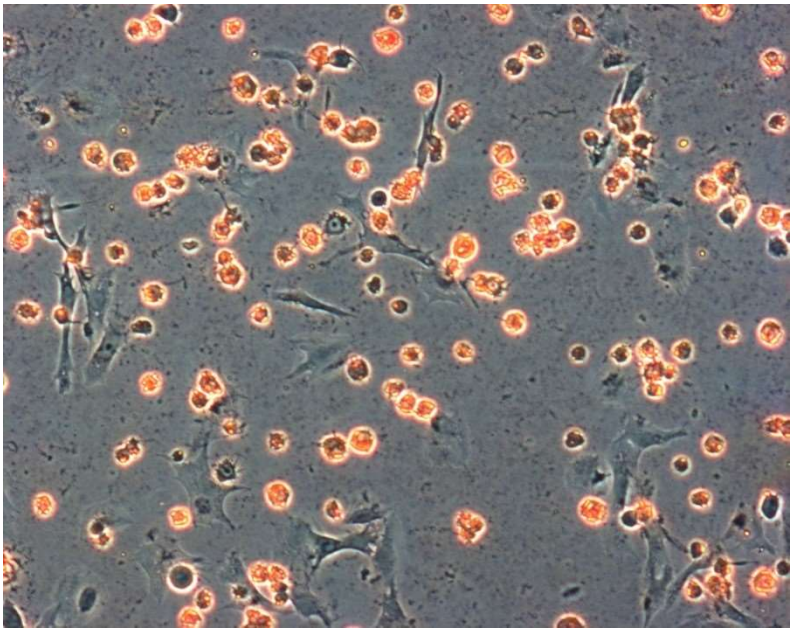

Figure 8.: Haemocytes of *B. mori*. Orange cells are reactive with the Bp4 monoclonal antibody

Ad 4.: **Recording and sampling of local mulberry varieties:**
The geographical locations of old mulberry trees have been identified in Hungary as well as in Slovenia and a distribution maps were created. In Hungary 1498 locations of *Morus alba* trees were collected during the field excursions. The highest density of trees was determined in Csongrád (226), followed by Bács-Kiskun (206), Somogy (180), Baranya (169), Veszprém (200), Zala (146), Hajdu-Bihar (128), Győr-Moson-Sopron (120), Szabolcs-Szatmár-Bereg (97) Tolna (83), Vas (70), Fejér (62), Békés (37), Heves (31), Pest (26), Borsod-Abaúj-Zemplén (16), Budapest (10) and Jász-Nagykun-Szolnok (1). Trees with highest circumference (max. 561 cm) were determined in Tolna region). The digital map of historical Hungarian and Slovenian genotypes is available on our project website: http://murve.um.si/en/home/; http://murve.um.si/en/maps/
The evaluation of the existing genetic resources in Slovenia revealed 643 mainly historical *M. alba* trees with the highest number of 436 trees in Submediterrean (SM) region, followed by Subpannonean (SP) region where 159 trees were documented. In addition, 29 trees were sampled in South-East (SE) region and 19 in Central (C) region (**Figure 9**.). The highest number of old mulberry trees with circumferences over 300 cm were determined in SM and C regions, which confirms that moriculture in Slovenia started in the areas close to Italy and was later spread towards other regions. Trees with circumferences between 200 and 299 cm were most common in SM region (34%), followed by trees of SP (27%), C (25%) and SE (14%) regions. Trees with circumferences between 100 and 199 cm were represented by 72% in SE region, whereas in other regions the percentage of smaller trees was less than 50%.
Pruning recordings showed that pollarding is traditionally used in SM region, since 31% of trees underwent yearly pollarding procedure, 46% of trees were pruned at least once in three years for crown reduction, whereas 23% of studied individuals were unpruned. In inner regions, the tradition of pollarding is no longer present. In the C and SE regions, 75% of sampled trees were left unpruned, whereas in the SP region the percentage of unpruned trees was 91% (Figure 9.).

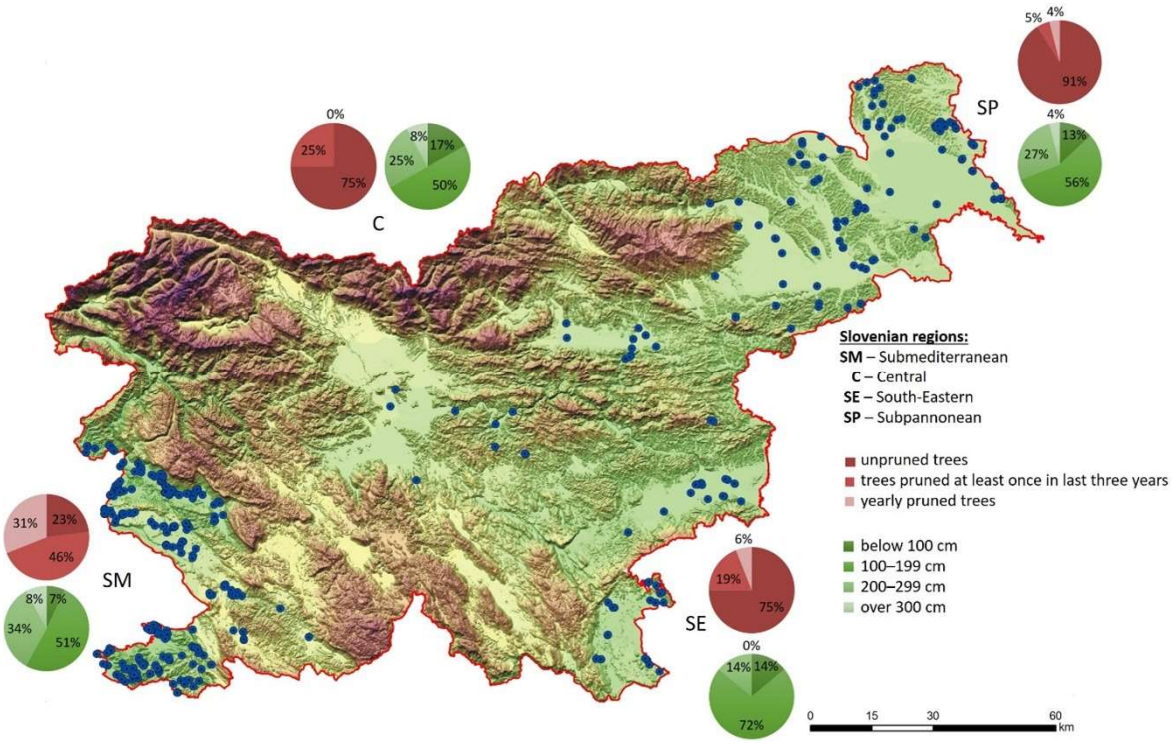

Figure 9.: Eco-geographical distribution of sampling localities of historical white mulberry (*Morus alba* L.) trees in Slovenia with attached trend diagrams of tree circumference and pruning frequency.

Ad 5.: **Propagation and conservation of mulberry varieties:**
Branches from the above-mentioned trees have been collected. Appropriate methods for the vegetative propagation of the cuttings were developed. Approximately 1200 plantlets were obtained comprising Hungarian and Slovenian historical mulberries. Mulberry gene bank (Moretum) was established at the Faculty of Agriculture and Life Sciences, University of Maribor (lat. 46.508459, long. 15.622440). The collection is divided into three parts: First part contains old high yielded mulberry varieties (8 varieties, N=80) brought from CRA-API Padova. In sericulture most

commonly, used varieties are ‘Morettiana’ (origins from India) and ‘Kokusou’ (origins from Japan), ‘Florio’ and ‘Giazzola’.
Second part is collection of old local Hungarian (N=255) and Slovenian mulberry varieties (N=133). Rooting of cuttings took place in nursery, however cuttings were planted in the collection when they reach about 70 cm height. The collection is still in establishment, there are around 400 plantlets which need to be planted into the Moretum in autumn.
Third part of collection contains mulberry varieties for fruit production. Until now 19 fruit trees were planted by obtaining the grafting material from different European gene banks and collectors. They are mostly crossbreeds between white and red mulberries (*M. alba* × *M. rubra*) from different origin (Romania, Ukraine, Bulgaria, France, Austria). In the collection there also two black mulberry trees (*Morus nigra*) derived from historical trees, one originates from eastern Austrian Styria and the other from Albania (village Boboshtice, mother plants are believed to be 1000 years old but probably between 400-500 years).
The collection has been used for screening of the metabolites with respect to the seasonal variation, pruning and production techniques and used for the feeding experiments on silkworms. Several trees were also identified as suitable for silkworm feed as they are characterized by large leaves and superior composition of metabolites.
More than 500 mulberry genotypes in the collection, that were sampled in different regions of Slovenia and Hungary was evaluated according to FAO descriptors. For leaf morphometrical analyses, eight leaf morphometrical parameters were measured, including leaf area, peduncle length, leaf length, leaf width, left and right leaf width from the rachis, and length of left and right basal vein. The statistical evaluation allowed us to establish the most suitable differentiating traits and to define closer morphotypes, and to present correlations between measured parameters with respects to pruning and geographical distribution. The results are published in Urbanek Krajnc et al. 2019: Morphometric and biochemical screening of old mulberry trees (*Morus alba* L.) in the former sericulture region of Slovenia. Acta Soc Bot Pol. 2019;88(1):3614. https://doi.org/10.5586/asbp.3614 Because the Slovenian partner was the corresponding author of the publication, the Slovenian identifier of the bilateral project (ARRS N1-0041) was indicated as financial source of the study.

Ad 6.: **Molecular characterization of local mulberry genotypes:**
Selected historical Slovenian and Hungarian mulberries were evaluated using six microsatellite markers (SSR) and the genetic relationships of these genotypes were compared with reference sericultural and modern varieties. The genetic characterization has been found sophisticated, mainly due to the different ploidy levels, sexual dimorphism, distribution in the past and natural genetic recombination among trees of different origins. Considering the traditional varieties, the clustering method grouped the genotypes into seven main clusters. The relatively high level of polymorphism reflects that in the past mulberries were mainly propagated generatively, and that they were distributed throughout the Austro-Hungarian countries. The study enabled us to reconstruct the origin and spread of mulberries among the silkworm rearers in the past.
Based on the multivariate analysis, we obtained a dendrogram in which Slovenian mulberry genotypes are classified into seven main groups. Polyploid genotypes were included in C1, among which the sample from Hrastovec is genetically related to the cultivar 'Agata', and some mulberries from the SP and SE region to the cultivar 'Shell'. In C2 we placed diploid historical trees of Školj Castle (Karst), which are genetically close to some mulberries of Koper Brda, Podgorski Kras and Rašica. Vipava mulberries (Selo) are closely related to C3 (Grad, Žetale, Stara Gora). In C4, there are mulberries that are genetically closely related to the older variety ‘Morettiana’; related mulberries of exceptional size are Fabiani's mulberry, Abitanti, Zazid, Miren, Seča (SM) and Goričko mulberry in Prosenjakovci (SP). C5 includes the tetraploid mulberries of the Benedictine monastery on Krog (SM), Dragatuš Castle (JV), Slovenske gorice (SP). Tetraploid mulberries from Vangenel (SM) and Adlešiči (JV) were placed in C6. In C6 we placed mainly Bela Krajina and Goričko mulberries, which are closely related to the two mulberries of Koper Brda.
Hungarian genotypes were also classified into seven main groups. Some mulberries have been shown to be closely related to the polyploid varieties ‘Japan Fuji Red’ (Békés), ‘Agate’ (Pest), ‘Kokusou’ (Veszprém), and the varieties ‘Florio’ (Győr-Moson-Sopron, Vas, Békés) and the older variety ‘Morettiana’. The oldest tree from Borsod-Abaúj-Zemplén and the thickest tree from Tolna are placed in the same separate diploid group. A separate main group consists of samples from the VE region, among which the historic tree-lined avenue of the Tótvázsony and Garabonc Calvinist cemeteries is worth mentioning. We confirmed four black mulberries in Slovenia and one in Hungary. In SM (Miren) and Baranya, we genetically confirmed *Morus australis*.
The article is in preparation, the results will be presented at the international scientific conference Slovenian symposium on Plant Biology in September 2022.

Ad 7.: **Biochemical analyses of primary and secondary metabolites of mulberry:**
For biochemical analyses, five to seven fully developed sun-exposed leaves were collected randomly from each tree and used as one sample. Total proteins were determined spectrophotometrically. In superior mulberry genotypes, protein contents exceeded 20 g / 100 mg DW. For analysis of single amino acids a HPLC method with OPA (o-phthalaldehyde) reagent precolumn derivatization was established. The total amount of phenolic compounds was determined using the Folin-Ciocalteu method. The methanol (95%) extracted samples were further analysed for single phenolic compounds by a gradient HPLC method.
Of the free amino acids, threonine, arginine, asparagine, serine and glutamine were the most predominant, which have proven antibacterial activity and have significant impact on the growth and development of silkworms. In the presented study, arginine and asparagine reached the highest average concentration in samples from Ajdovščina. The results of the pruning impact study clearly show that yearly prunned trees have higher asparagine, alanine and serine contents compared to unprunned trees (**Figure 10a**).
The main phenolic compounds identified in leaves were caffeoylquinic acid derivatives, quercetin malonylhexoside, rutin, kaempferol acethylhexoside and isoquercetin. Pruning significantly affected the levels of total protein and quercetin dirhamnosylhexoside, which were higher in the annually pruned trees. Geographical distribution has a significant effect on total proteins as well as on total phenolics, caffeoylquinic acid derivatives and flavonols (**Figure 10b**).

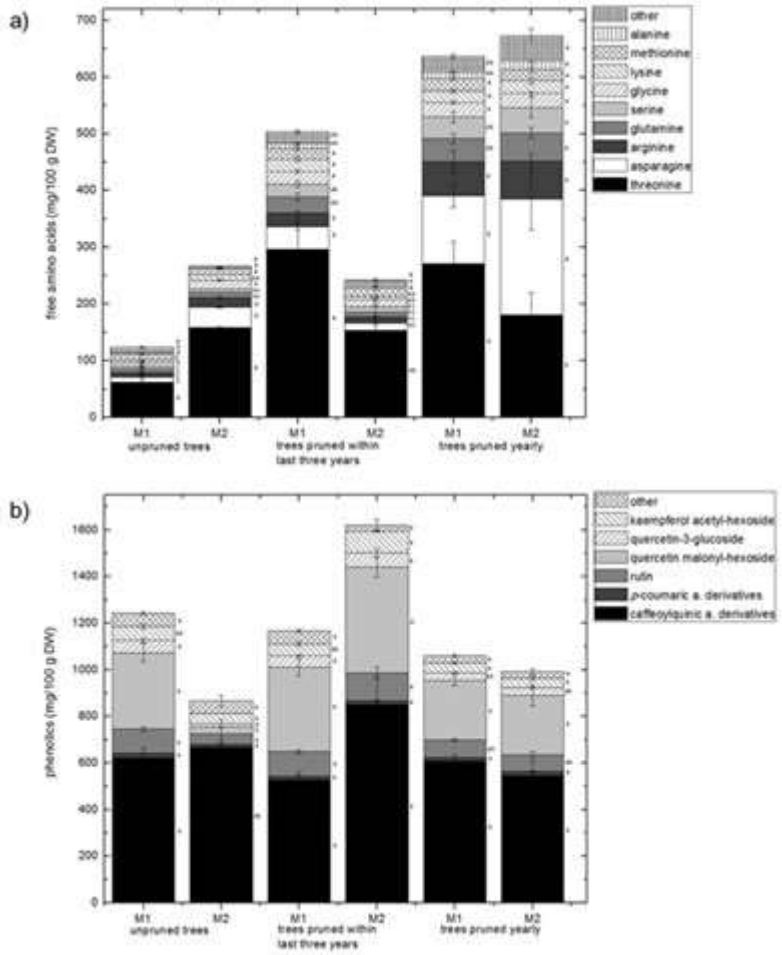

Figure 10: Content levels of predominant a) amino acids and b) phenolics within both morphotypes (M1, M2) with respect to pruning management.

Each value represents mean ± SE of each component within each pruning management/ morphotype category. Different letters (a–b) indicate significant differences ($P < 0.05$), which were determined using the post-hoc Duncan test.

Geographical distribution has a significant effect on total phenolics, caffeoylquinic acid derivatives and flavonols (**Figure 11.**). Hydroxycinnamic acids with the predominant chlorogenic acid were highest in SM region and lowest in SP region. Flavonols with the predominant quercetin dirhamnosylhexoside were highest in SE region (Figure 11.).

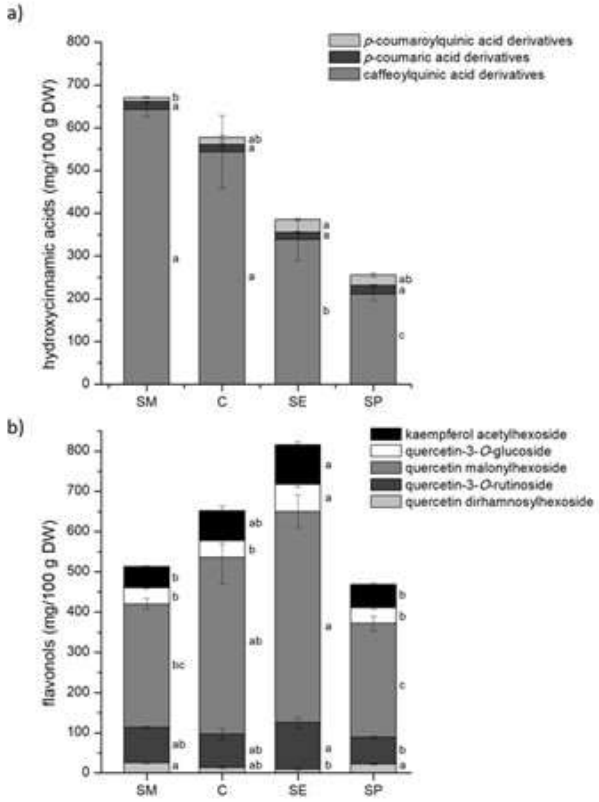

Figure 11.: Content levels (mean ± SE) of identified a) hydroxycinnamic acids and b) flavonols in mulberry leaves in association with eco-geographical regions.
Different letters (a–c) indicate significant differences ($P < 0.05$), which were determined using the post-hoc Tukey test.

Linear discriminant analysis enabled a comprehensive assessment of leaf metabolites and confirmed that mulberries of different Slovenian regions have distinctive biochemical traits (**Figure 12.**).

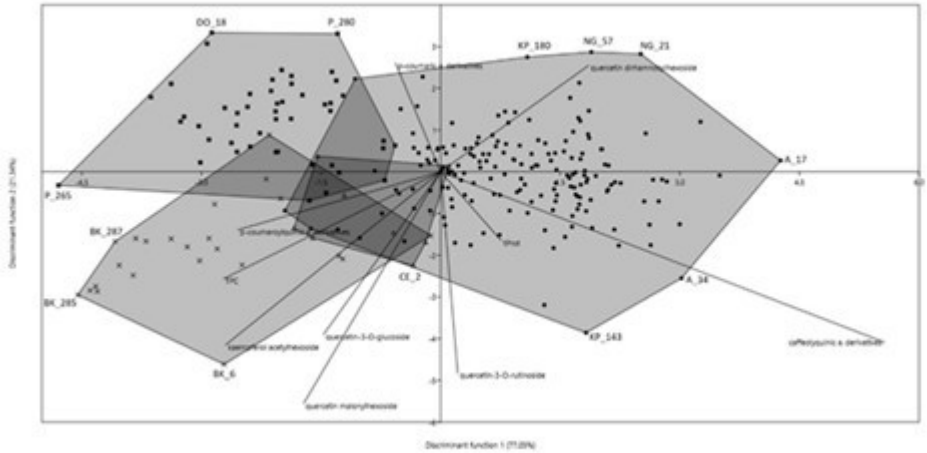

Figure 12.: Distribution of the first two discriminant functions according to linear discriminant analysis (LDA) of different biochemical traits in *M. alba* leaves, with marked superior genotypes.
The convex hulls delimit the space that includes the samples of mulberry trees from different Slovenian regions. Trees of SM, SP, SE and C regions are marked with dots, squares, crosses and triangles, respectively.

Multivariate analyzes allowed us to define seven chemotypes in more detail in terms of the composition of individual amino acids and phenolics (**Figure 13.**). We were able to recognize caffeoylquinic acid, quercetin malonylhexoside, rutin, kaempferol acetylhexoside and isoquercetin as valuable feed markers with respect to their known beneficial effect on silkworm larvae growth and cocoon quality (Figure 12.). By reviewing the existing mulberry genetic resources and their leaf metabolites, we highlighted the natural, cultural and scientific value of the white mulberry. The analyses presented in the article were used as basis for the silkworm feeding experiment.

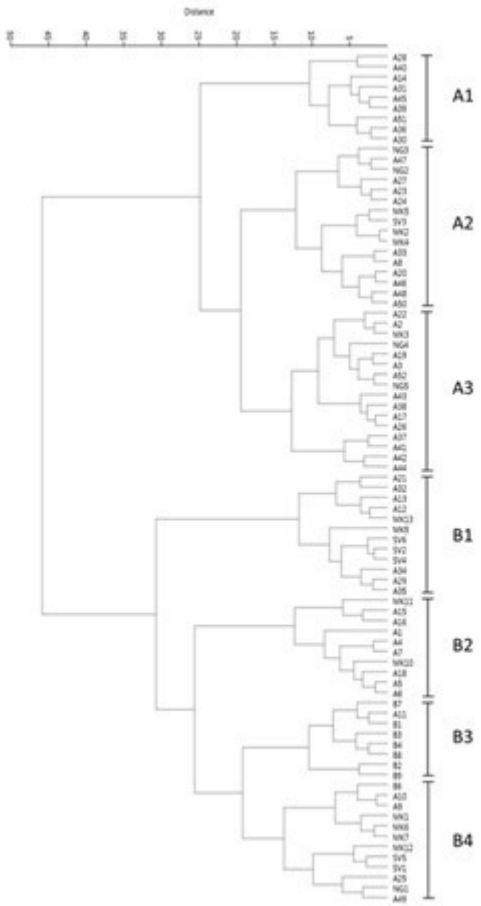

Figure 13.: Dendrogram combining the analysed morphometrical and biochemical traits of studied local mulberry genotypes, using hierarchical clustering method based on Euclidian distance.

The results of the study were published in Urbanek Krajnc et al. 2019: Morphometric and biochemical screening of old mulberry trees (*Morus alba* L.) in the former sericulture region of Slovenia. Acta Soc Bot Pol. 2019;88(1):3614. https://doi.org/10.5586/asbp.3614 and in Šelih et al. (2020): Screening of leaf metabolites in historical mulberry trees (*Morus alba* L.) from different eco-geographical regions of Slovenia. Trees. 2020;34(4):971-986. https://link.springer.com/article/10.1007%2Fs00468-020-01974-z. Because the Slovenian partner was the corresponding author of the publication, the Slovenian identifier of the bilateral project (ARRS N1-0041) was indicated as financial source of the study.

Ad 7.: **Experimental feeding of silkworms with leaves of selected mulberry trees:**
The controlled feeding experiment was implemented in the 2021 silkworm rearing season. Eggs of a silkworm hybrid, which is frequently used for cocoon production in Northern Italy were provided by CRA-API. Newly hatched larvae were randomly divided into 61 groups containing 30 individuals each. Larvae in the groups were fed with contained different of leaves collected (e.g. from 16 Hungarian, 10 Slovenian mulberry genotypes and 8 “exotic” (Italian, fruit selected) varieties grown in the gene bank, 18 groups feed with the same genotypes grown in different regions of Slovenia and Hungary, etc). The leaf consumption and body weight gain were measured and documented on daily basis. The growth of the larvae was synchronised by harmonised feeding reintroductions after moulting. One group was fed with 50% amount of the average consumption of leaves in the other experimental groups. This group was established to model the effect of restricted access to food. The activity and health status of the larvae was

continuously monitored. Signs of diseases were not observed in any groups and very little larval death was observed in certain experimental groups during the first four instar stages. In the fifth instar stage, marked differences were observed in the body weight gains, in-group homogeneities and spinning activities of the larvae. In general, more that 90% of larvae successfully spined in the groups fed with Hungarian, Slovenian and sericulture-selected "exotic" mulberry genotypes, but some of those that were fed with leaves of fruit production selected mulberries failed spinning by >20%. (E.g. larvae fed with *M. nigra* leaves gained weight over the average, but none of them produced cocoons.) The cocoons were collected, dried and was submitted for quality analysis to the Italian partner (to apply standard, unbiased evaluation of the cocoon and rope quality). The average weights of the dried cocoons did not differ considerably (0.96 g of the Slovenian and 1.02 g of the Hungarian and exotic genotypes.) Preliminary results on the analysis of leaf contents indicate strong correlation between rutin, kaempferol acethylhexoside and isoquercetin and the weight of 5th C/5-7th D silkworms, whereas there was a weak correlation between silkworm weight and quercetin malonyl hexoside. A medium correlation was found between total phenolics, quercetin malonyl hexoside and kaempherol-acethyl-hexoside and the fresh cocoon weight (**Table 1.**).

Table 1.: Pearson correlation coefficients between analyzed mulberry leaf biochemical traits.

	5th C/5th D - 1 worm (g)	5th C/6th D - 1 worm (g)	5th C/7th D - 1 worm (g)	1 cocoon - fresh (g)	total proteins (mg/g DW)	total phenolics (mg/g DW)	chlorogenic acid (mg/g DW)	4-caffeoquinic acid (mg/g DW)	5-coumaroylquinic a. cis (mg/g DW)	quercetin-3-O-rutinoside (mg/g DW)	isoquercetin (mg/g DW)	quercetin malonylhexoside (mg/g DW)	kaempferol acetylhexoside (mg/g DW)
5th C/5th D - 1 worm (g)		0,000	0,000	0,000	0,849	0,848	0,251	0,505	0,486	0,370	0,894	0,153	0,972
5th C/6th D - 1 worm (g)	0,000		0,000	0,000	0,748	0,946	0,506	0,745	0,659	0,901	0,761	0,433	0,890
5th C/7th D - 1 worm (g)	0,000	0,000		0,000	0,988	0,981	0,841	0,576	0,943	0,952	0,736	0,224	0,759
1 cocoon - fresh (g)	0,000	0,000	0,000		0,310	0,350	0,009	0,138	0,029	0,015	0,178	0,514	0,412
total proteins (mg/g DW)	0,849	0,748	0,988	0,310		0,000	0,000	0,001	0,000	0,002	0,005	0,000	0,003
total phenolics (mg/g DW)	0,848	0,946	0,981	0,350	0,000		0,000	0,000	0,249	0,004	0,002	0,000	0,006
chlorogenic acid (mg/g DW)	0,251	0,506	0,841	0,009	0,000	0,000		0,018	0,000	0,000	0,000	0,000	0,000
4-caffeoquinic acid (mg/g DW)	0,505	0,745	0,576	0,138	0,001	0,000	0,018		0,074	0,011	0,446	0,628	0,946
5-coumaroylquinic a. cis (mg/g DW)	0,486	0,659	0,943	0,029	0,000	0,249	0,000	0,074		0,130	0,167	0,010	0,000
quercetin-3-O-rutinoside (mg/g DW)	0,370	0,901	0,952	0,015	0,002	0,004	0,000	0,011	0,130		0,000	0,002	0,025
isoquercetin (mg/g DW)	0,894	0,761	0,736	0,178	0,005	0,002	0,000	0,446	0,167	0,000		0,000	0,001
quercetin malonylhexoside (mg/g DW)	0,153	0,433	0,224	0,514	0,000	0,000	0,000	0,628	0,010	0,002	0,000		0,000
kaempferol acetylhexoside (mg/g DW)	0,972	0,890	0,759	0,412	0,003	0,006	0,000	0,946	0,000	0,025	0,001	0,000	

All selected Hungarian and Slovenian genotypes gave cocoons of the highest quality when compared to reference sericultural varieties. The analysis enables us to select superior mulberry genotypes in respect to silkworm weight and cocoon yield (**Table 2.**).

Table 2.: Average values of silkworm weight 5th C/5-7th D, cocoon weight and individual biochemical compounds.

SAMPLE	5th C/5th D - 1 worm (g)	5th C/6th D - 1 worm (g)	5th C/7th D - 1 worm (g)	1 cocoon - fresh (g)	total proteins (mg/g DW)	total phenolics (mg/g DW)	chlorogenic acid (mg/g DW)	4-caffeoquinic acid (mg/g DW)	5-coumaroylquinic a. cis (mg/g DW)	quercetin-3-O-rutinoside (mg/g DW)	isoquercetin (mg/g DW)	quercetin malonylhexoside (mg/g DW)	kaempferol acetylhexoside (mg/g DW)
Italian old high yielded mulberry varieties													
MORETT. (23.6)	4,350	4,670	5,110	2,170	182,940	18,750	7,440	0,926	0,775	3,221	0,517	1,557	1,347
KOKUSOU (23.6)	3,910	4,760	5,590	2,200	205,917	21,360	17,666	0,895	1,678	4,101	0,541	2,249	3,203
FLORIO (23.6)	4,850	5,070	5,310	2,340	197,920	20,540	13,273	1,225	1,135	3,101	0,265	1,832	2,511
GIAZZOLA (23.6)	4,420	4,440	4,830	2,200	178,721	19,150	8,485	1,078	0,904	2,642	0,237	0,879	1,281
Slovenian mulberry varieties													
PT 304 (23.6)	4,220	4,360	5,140	2,100	214,569	16,823	8,687	1,021	1,666	2,302	0,222	1,372	2,335
BK 8 (23.6)	4,310	4,500	5,030	2,270	217,926	16,710	9,662	0,731	1,708	1,941	0,238	1,570	2,156
BK 5 (23.6)	3,910	4,370	4,740	2,200	222,139	19,347	11,731	0,885	1,091	3,161	0,340	1,638	1,721
P/137 (23.6)	3,460	3,810	4,190	2,110	211,042	15,970	7,759	0,737	1,450	2,851	0,367	1,311	2,174
P256 (23.6)	4,040	4,350	4,880	2,080	186,994	18,530	8,789	0,790	0,907	1,969	0,196	1,655	2,259
MB 303 (23.6)	3,930	4,110	4,470	2,010	229,609	17,410	10,418	0,885	1,842	1,860	0,278	1,557	2,701
S 100.1 (23.6)	3,220	3,920	4,340	2,070	239,417	17,543	8,444	1,064	1,600	2,945	0,293	1,926	2,043
SG 12 (23.6)	4,660	4,780	5,470	2,350	188,591	11,802	9,145	0,240	1,888	2,032	0,181	1,511	3,220
NG 214 (23.6)	4,430	4,660	5,260	2,19 g	192,997	16,215	7,487	1,178	1,297	1,338	0,261	1,012	1,452
Hungarian mulberry varieties													
SO 1035 (23.6)	3,990	4,170	4,470	2,230	220,604	18,618	13,519	0,787	2,327	2,311	0,311	1,959	3,260
SO 1013 (23.6)	4,120	4,190	4,680	2,200	221,887	16,950	9,627	0,925	1,799	2,383	0,217	1,264	1,780
BE 1264/2 (23.6)	4,140	4,450	4,840	2,260	225,901	17,840	10,479	0,939	1,797	3,097	0,291	2,080	3,172
ZA 1060 (23.6)	4,810	4,970	5,370	2,300	226,891	19,325	11,266	0,637	1,082	2,481	0,453	2,029	2,715
ZA 1070 (23.6)	3,990	4,410	4,910	2,310	210,185	21,030	10,477	0,976	1,077	3,181	0,321	1,877	1,530
ZA 2084 (23.6)	4,360	4,720	5,295	2,190	202,883	18,108	7,805	0,917	1,187	1,914	0,258	1,557	2,622
TO 1131 (23.6)	5,200	4,800	5,320	2,450	212,697	18,870	10,657	1,366	2,001	4,307	0,368	1,386	2,270
VE 2706 (23.6)	3,470	3,700	4,040	2,180	196,712	19,040	8,804	0,660	0,870	2,697	0,364	2,048	2,872
GMS 2286 (23.6)	4,180	4,410	5,000	2,230	215,789	17,577	9,089	1,046	1,575	2,931	0,351	1,476	1,889
GMS 2329 (23.6)	4,450	4,720	5,010	2,380	219,846	17,160	11,094	0,918	1,955	2,306	0,303	1,777	1,927
GMS 2532 (23.6)	4,830	4,490	5,400	2,290	210,486	18,805	9,997	1,258	1,058	3,057	0,186	0,994	1,556
GMS 2533 (23.6)	4,380	4,420	4,820	2,190	178,806	17,875	9,174	1,221	0,966	2,681	0,166	0,866	1,326

Samples were collected from healthy and ill/death larvae and were tested for the presence of BmNPV by real-time PCR. Viral nucleic acid was detected in several samples, including clinically healthy larvae, however in much lower concentrations compared to the ill and/or dead ones (see Ad 1.). The gut microbiome was also determined in larvae of selected groups. Comparative analyses of the data are ongoing at the time of writing the final scientific report of the project. We plan to publish the results of this study in a scientific article.

Conclusions and further perspectives

In this bilateral cooperation between the Hungarian and Slovenian partner we have successfully integrated the veterinary microbiology and botany expertise as a comprehensive approach involving plant physiology, microbiology and animal husbandry. Despite the technical difficulties and delays during implementation, the research resulted several new scientific information on the diversity and biochemical characteristics of mulberries, the physiology, immune mechanisms and gut microbiome composition of the silkworm, as well as certain characteristics of BmNPV, which was found as the most relevant pathogen of *B. mori* in the European environment.
The project resulted additional, practical achievements. Within the last six years, extensive theoretical and practical knowledge was gained on moriculturing, sericulturing and infectious diseases of the silkworms. Additionally, the activity was advertised within the two countries and interested volunteers had the possibility to learn sericulture practices. Some farmers have reared silkworms in Slovenia and in Hungary in consecutive years and with success. The established Moretum with biochemically characterised mulberry genotypes can provide sources for the reintroduction of moriculture in the countries. The available knowledge, expertise and diagnostic capacities provide sufficient support for the re-establishment of sericulture in Central Europe.