To METHAGENE COST Short Term Scientific Mission officials

SHORT SCIENTIFIC REPORT of the STSM of dr. Zala Prevoršek at Helmholtz Zentrum Munich
(COST STSM Reference Number: COST-STSM-FA1302-24634)

Short term scientific mission in Helmholtz Zentrum Munich (February 1st – March 2nd, 2015)

DNA-RNA coextraction from rumen samples and MiSeq sequencing of mRNA transcripts

The objective of our study is to identify and record the metatranscriptome of the realistic microbial communities in the gastrointestinal tract of five species of slovenian alpine ruminants (roebuck, deer, mouflon, capricorn, chamois) that are characterized by high efficiency in using a variety of plant polymers, to assess their intertemporal connectivity and dependence on the type of plant polymers and their degradation products, focused on methane production. Direct sequencing of transcriptome of the complete sampled microbial community and transcriptomes from timeline samples in experiments with natural complex plant polymer substrates is going to provide means to determine transcripts that are related to substrate characteristics, experimental conditions and rumen methane production in wild alpine herbivores.

Trancriptome analysis in relation to measured production of methane in particular time slices of experiments is going to show which transcripts are most likely responsible for production of methane in the rumen, and concomitant degradation of specific plant polymer components under anaerobic conditions.

Introduction

My name is Zala Prevorsek and I am a postdoctoral researcher at Biotechnical faculty, University of Ljubljana, Slovenia. Since 2005 I have been working in genetics laboratory of Chair of Genetics, Animal Biotechnology and Immunology, University of Ljubljana. My previous work was focused on mapping quantitative trait loci (QTLs) in mice, but in summer 2014 I changed my area of research by joining a research group of dr. Blaz Stres. We are currently working on a microbial project, which is focused on finding novel genes related to polymer degradation and methane production in complex microbial communities and their overlap with mRNA transcripts.

Methagene Short therm scientific mission (STSM)

The whole STSM was planned for duration of four weeks with the following general aims:

A1: To perform the DNA and RNA coextraction protocol on real samples with active degradation of complex recalcitrant organic materials
A2: To assess mRNA fraction through the time-series of samples and optimize its recovery.
A3: To obtain hands-on experience in MiSeq operation and data analysis.

Report, week 1 and week 2:

I arrived to Munich, Germany, on February 1st 2015. I brought the rumen samples with me, stored on dry ice, along with all necessary molecular kits, chemicals and extraction platforms. On 2nd of February I started working in the lab of Research Unit for Environmental Genomics, Helmholtz Zentrum Munich.

First, I preformed DNA and RNA coextraction from rumen samples of chamois, deer, roe deer and mouflon using Mobio Power Microbiome kits. After the extraction, I separated the nucleic acids into two aliquots; one was treated with DNAse while the other was treated with RNAse. Quantities of nucleic acids and their size distribution in both fractions were determined using Nanodrop and with gel electrophoresis.

The DNA and RNA yield was high, but I had problems with low A260/230 ratios, suggesting low RNA purity. I repeated the extractions, tried several modifications of the protocol to get better ratios, but they did not improve. The composition of the rumen samples might be the problem. After discussing this matter with fellows from the lab, who have more experience in this field of research, we decided to try and synthesize the cDNA despite low ratios.

On February 11th I presented my STSM at a research group lab meeting.

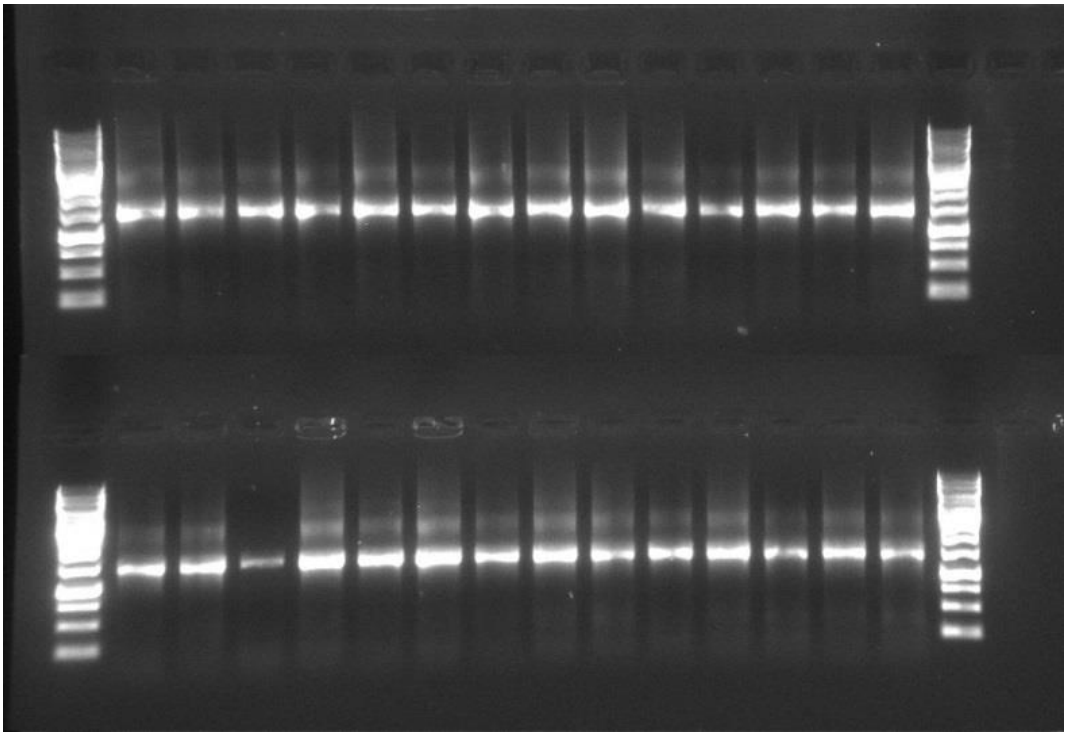
The reverse transcription of RNA fraction using random hexamer primers, was performed to obtain the cDNA, using High Capacity cDNA Reverse Transcription kit. Before proceeding, I checked every cDNA sample on gel electrophoresis. Based on the gel pictures, the mouflon cDNA was less concentrated than samples from other species, so I repeated the reverse transcription for all mouflon samples. cDNA was then visible on the gel, so we continued with the protocol.

Report: week 3 and week 4:

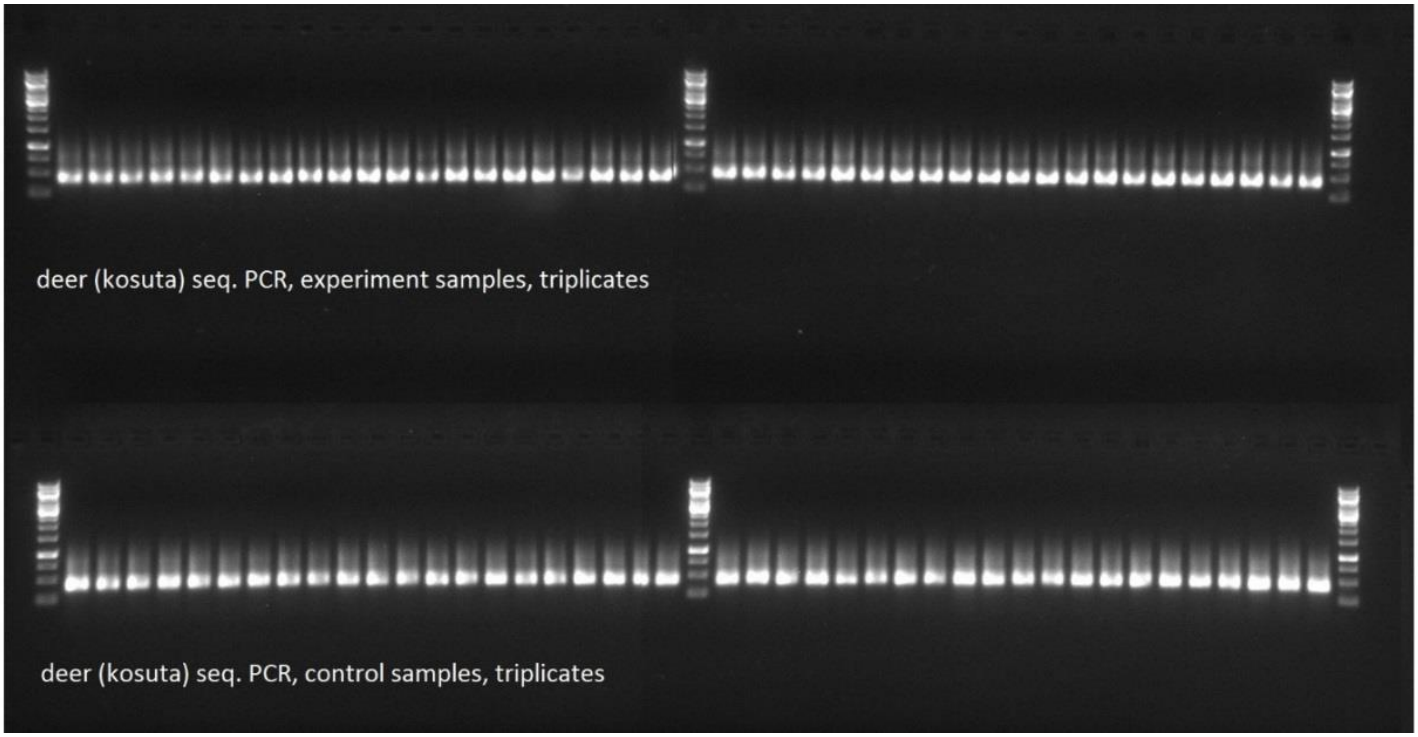
We started with the 16S Metagenomic Sequencing Library Preparation for the preparation of NGS-ready fragments of amplicon DNA derived from rumen samples. The analysis of prokaryotic 16s rRNA is a common method used to identify and compare bacteria present within a given sample. This method has been used to evaluate bacterial diversity in many environments, allowing researchers to characterize microbiomes from samples that are otherwise difficult or impossible to study.

We performed 16s rRNA PCR with universal primers primers 1401R in 27F, before proceeding to Illumina sequencing PCRs. 16s RNA PCR worked beautifully for all rumen samples.

16s rRNA PCR using deer rumen samples cDNA



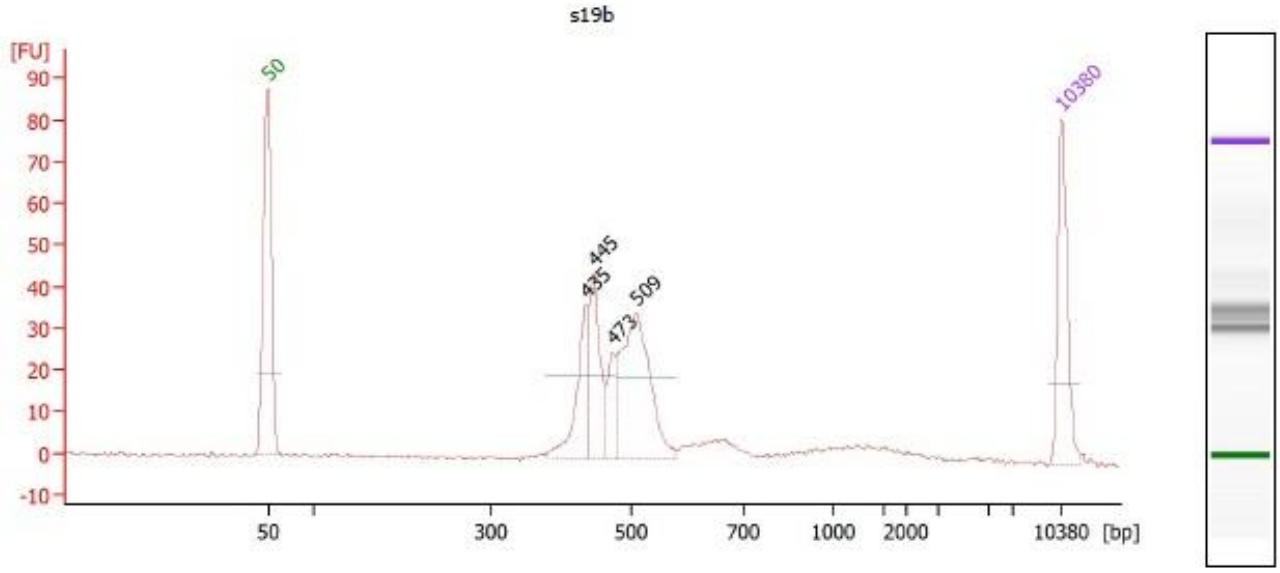
After I successfully synthesized the cDNA from all rumen RNA samples, I performed the Illumina sequencing PCRs and all samples worked beautifully, gel electrophoresis verified that fragments are the expected size and free of primer dimer.



All PCR reactions were then purified with NucleoSpin kit (to remove the dNTPs, primers ald primer dimers). A few random samples were quantified on the Bioanalyzer 2100. The readings confirmed that my samples are the expected size and clean of any primer dimers.

Example: Sample S19b quantification

Electropherogram Summary Continued ...



Overall Results for sample 2 : s19b

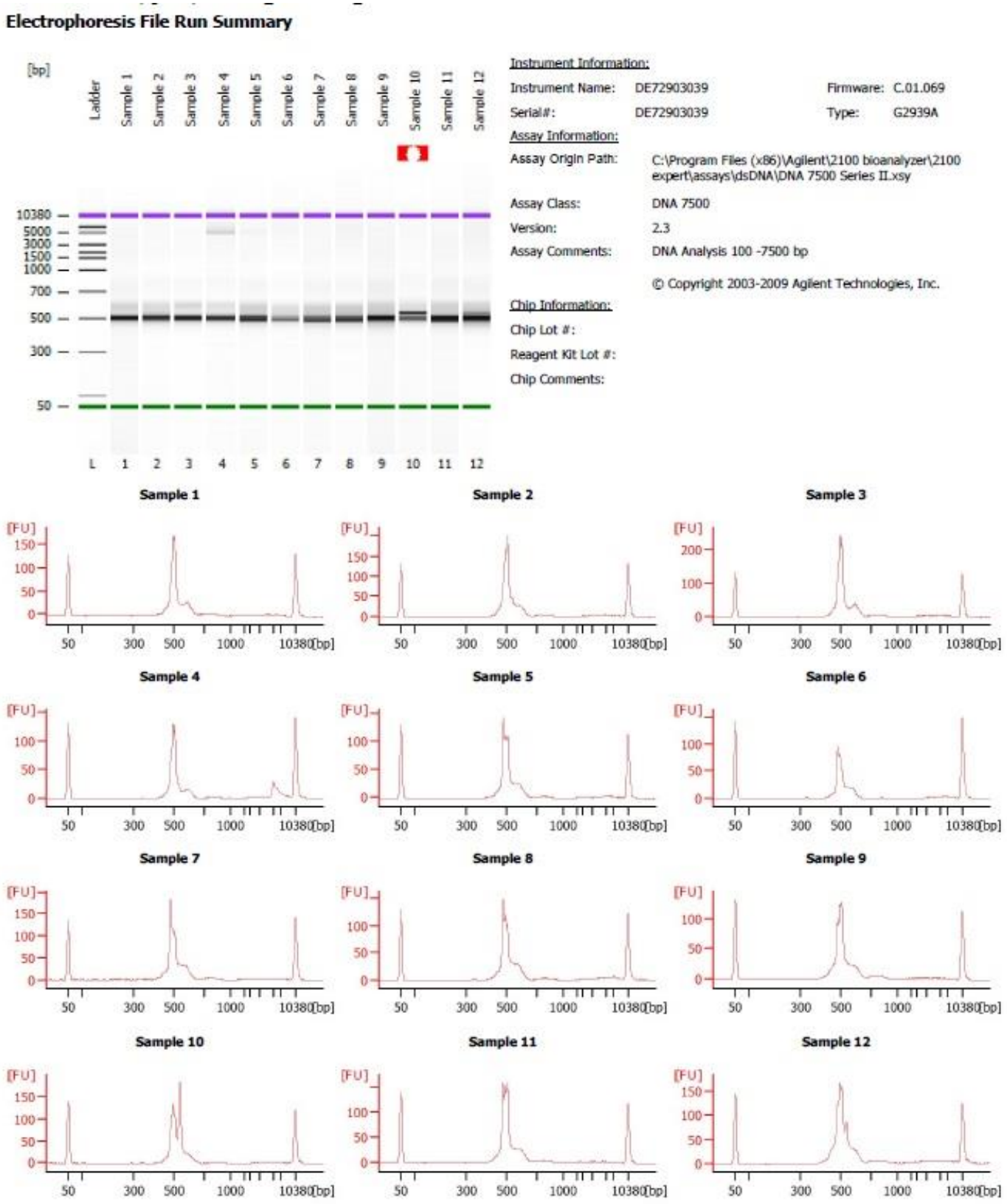
Number of peaks found: 4

Peak table for sample 2 : s19b

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2	435	2.77	9.6	
3	445	3.44	11.7	
4	473	1.35	4.3	
5	509	6.63	19.7	
6	10	4.20	0.6	Upper Marker

Before performing the indexing PCR, we quantified all the samples with fluorometric quantification using Picogreen dye. Based on the results, we diluted the DNA samples to concentration needed for the indexing PCR. The indexed DNA library for next-generation sequencing on the Illumina platform was prepared using NebNext Ultra, DNA Library prep kit.

Second Quantification on Bioanalyzer 2100: 12 random samples were quantified on Agilent Bioanalyzer 2100



Second fluorometric quantification of samples using Picogreen dye: My samples were then again quantified using a fluorometric quantification method using Picogreen dye to obtain the exact DNA concentrations.

Library Quantification, Normalization, and Pooling: Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes. I Calculated DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer: average library size = 500bp. Then I diluted samples to 4nM concentration. I aliquoted 5ul of diluted DNA from each library and pooled the libraries with unique indices. My samples were then prepared for MiSeq sequencing.

Conclusions and next steps

Anaerobic decomposition of organic polymers of plant origin and the production of methane in the digestive tract of alpine wild ruminants is extremely poorly understood system. These systems rely on decomposition of organic polymers with significantly lower content of readily biodegradable organic matter and a greater proportion of crosslinked lignocellulose structures and an increasing share of fungi and algae from lichens. The very first studies reporting on indepth analyses of microbial diversity of bacteria, archaea and protozoa in these systems were published only recently, but this only contributed to the understanding of microbial diversity and taxonomic status of the census in gastrointestinal tract. Functional aspects or genetic diversity of functional and housekeeping genes present in microbial communities of these systems has remained largely undescribed and untampered. No metagenomic study targetting genetic diversity of functional genes involved in metagenesis in the intestinal contents of alpine wild ruminants has been conducted so far, especially not in the context of precharacterized plant substrates.

Monitoring of transcriptome simultaneously with the degradation of recalcitrant plant polymeric substrates and methane production in factorial experiment in the rumen of wild ruminants have not been reported and thus represents an important step forward linking the occurrence sequence information with the complexity of measured environmental parameters, expanding set of known enzymes involved in production of methane and the decomposition of plant substrates, which are different from classical digestable industry oriented feed, which was studied only in the form of metagenomes (DNA level) so far, but could power the use of uncovered enzymes for industrial purposes to improve the process of depolymerization of lignocellulosic residues alone or in combination with already existing industrial enzyme preparations.

The first stage of our experiment started during the hunting season in autumn 2014, when we collected rumen samples of wild ruminants and performed the incubation experiment in which we measured the production of methane in its codependency with the degradation of different recalcitrant plant substrates using Automatic Methane Potential Test System II. Samples of intestinal contents were incubated until the major fraction of biodegradable organic polymers in the samples was anaerobically decomposed, generally 24 to 48 hours based on the passage rates in gastrointestinal tract of wild ruminants. Incubation was conducted in a temperature and gas controlled reactors at 39°C under anaerobic conditions. Decomposition of plant material in the input patterns was monitored.

During my 30 day STSM in Helmholtz Zentrum in February 2015 I successfully implemented the second stage of the experiment – I prepared 16S Amplicon DNA Library and metagenomic DNA library for next generation MiSeq Illumina sequencing for all 4 wild herbivore species as planned. MiSeq next generation amplicon an metagenomic sequencing has already been performed.

The next step of our study is sequence analysis. This is going to be performed within the MGRAST server (Meta Genome Rapid Annotation using Subsystem Technology), Argonne National Library, USA, that is dedicated to metagenomic analyses. MGRAST SEED Subsystems and presentation using KEGG Mapper are going to be used to view and reconstruct the entire metabolic pathways from metatranscriptomes and their comparison with known metagenomes available in databases. Statistical and multivariate analyses will be conducted in R.

In the near future, the rumen samples are going to be characterized by liquid state NMR to describe the formation of various metabolites from rumen samples. In the second phase solid state NMR spectra are going to be used to identify which organic groups are preferentially degraded during the incubation experiments and have become a source of metabolites detected by liquid state NMR. Within this experiment, we are also going to measure volatile fatty acids and sugars in the rumen content of wild apline ruminants.

Trancriptome analysis (in extension to cooccurrence and metabolic networks) in relation to measured environmental parameters is going to show which transcripts are most likely responsible for production / degradation of particular metabolites and concomittant degradation of specific plant polymere components and methane production under anaerobic conditions. Based on the fact that wild ruminants ingest hard-to - degrade plant materials relative to domesticated ones and therefore contain higher concentrations of various plant polymeric compounds, a more concise description of methane emmissions, metabolic conversions, microbial interactions and plant polyphenol derivatives in wild ruminants intestinal tract is going to providea valuable insight that would ultimately yield a better understanding of rumen functioning per-se (as to the best of our knowledge we are not aware of any comparable study) and also provide additional strategies in the form of novel plant polymeric compounds from alpine altitude feed for mitigation of methane emissions to be tested in domesticated ruimants in the future.

We expect to submit at least one scientific publication based on the results of my work in HHZ and Methagene COST wil be acknowledged. We also plan to present the results on several conferences in the near future.

At the end of my very successful STSM I would like to thank the METHAGENE COST Action for their support and for making my STSM possible.

Domžale, Slovenia, 17.3.2015