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STUDENT PERFORMANCE STUDY: THE OUTCOMES OF METABOLIC, MOLECULAR AND PHYSICAL-CHEMICAL CHARACTERIZATION OF INTESTINAL TRACT MICROBIOME ON A FOUR MAMMALIAN SPECIES MODEL

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Student performance study: the outcomes of metabolic, molecular and physical-chemical characterization of intestinal tract microbiome on a four mammalian species model

Many environmental factors influence the structure of microbial communities, their activity and properties of the environment of the digestive tract. Contrary to constant disturbances, the system provides the basis for energy conversion and thus the long-term stable coexistence of different hosts and their specific intestinal microbiota over geological timescales. Since the methodological approaches proved to be the largest source of systematic errors in comparisons of microbial communities among different organisms of the same species or between different species, we tested a number of methods on samples from different species of mammals in order to verify the feasibility of this approach for future routine analysis of microbiomes:(i) analyses of physical-chemical parameters;(ii)the metabolic properties of attached, planktonic fractions in comparison to the total;(iii)structure of microbial communities of bacteria and archaea; (iv)data analysis. We used a model of intestinal samples from four species of mammals, encompassing the differences between the various types of intestinal tracts: ruminants and rodents (such as pre- and post- peptic fermentors), omnivores and carnivores. The second purpose of the study was to(i)assess the extent of spread of data due to the cooperation of the various operators on the data obtained, and(ii) to evaluate the skills of the students to carry out industry-oriented investigations and measurements in 1st year of MSc study Microbiology; and(iii) to promote awareness of the importance of routine laboratory work day and the corresponding duties. The results suggest(i)that the operators independently organized and shared tasks;(ii)successfully completed all methods;(iii)obtain relevant information;(iv)critically evaluated and interpreted within the extent of their knowledge;(v) that relative standard deviation(RSD) typically could be compared to those of the automated analytical procedures(<10 %) and therefore represented the maximum extent of the variability of the biological material itself. It follows that the motivated MSc students were able to uphold the unknown protocols under supervision and perform laboratory and analytical complex experimental task, process and interpret results, and approximate performance of analytical procedures in industrial laboratories to generate data sets of acceptable high-quality.

Key words: microbiology / mammals / intestinal tract / microbiota / metabolic profiling / student work / quality

Ocena študentske uspešnosti: rezultati metabolnih, molekularnih in fizikalno-kemijskih karakterizacij mikrobiomov prebavnega trakta na modelu štirih vrst sesalcev

Številni okoljski dejavniki vplivajo na strukturo mikrobnih združb, njihovo aktivnost in lastnosti okolja prebavnega trakta, vendar ne glede na večino sprememb sistem zagotavlja podlago za pretvorbo energije ter s tem dolgoročno stabilni soobstoj različnih gostiteljev in lastne specifične črevesne mikrobiote preko geoloških časovnih okvirov. Ker so se metodološki pristopi izkazali za največji vir sistematičnih napak v primerjavah mikrobnih združb med različnimi organizmi iste vrste ali med različnimi vrstami, smo preizkusili številne metode na vzorcih iz različnih vrst sesalcev z namenom preveriti izvedljivost takega pristopa za prihodnje rutinske analize mikrobiomov: (i) analize fizikalno-kemijskih parametrov; (ii) metabolne lastnosti pritrjene planktonske frakcije v primerjavi s celokupno; (iii) strukture mikrobnih združb bakterij in arhej; (iv) analitske pristopke k analizi podatkov. Uporabili smo modelne vsebine prebavil štirih vrst sesalcev, ki zajema v veliki meri razlike med različnimi tipi prebavil: prežvekovalci in glodalci (kot pre- in post- peptični fermentatorji), vsejede ter zveri. Drugi namen študije je bil (i) oceniti obseg raztrosa podatkov zaradi sodelovanja različnih operaterjev

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na pridobljene podatke ter (ii) ovrednotiti usposobljenost skupine študentov za opravljanje industrijsko orientiranih preiskav in meritev v 1.letniku magistrskega študija Mikrobiologija, ter (iii) spodbuditi ozaveščenost o pomembnosti in pomenu aktivnosti s simulacijo rednega laboratorijskega delovnega dneva in pripadajočih dolžnosti. Rezultati kažejo, (i) da so se operaterji samostojno organizirali in razdelili naloge; (ii) uspešno izvedli vse metode; (iii) pridobili relevantne podatke; (iv) jih kritično vrednotili in interpretirali v obsegu svojega znanja; (v) da so raztrosi podatkov (RSD) tipično primerljivi s tistimi iz avtomatiziranih analitskih procedur (<10 %) in ponazarjajo v največji meri variabilnosti samega biološkega materiala. Iz tega izhaja, da so se motivirani magistrski študenti ob ustreznem vodenju sposobni spoznati z neznanimi protokoli ter izvesti laboratorijsko in analitsko kompleksne eksperimentalne naloge, rezultate obdelati, izsledke interpretirati, ter se približati izvajanju analitskih procedur v industrijskih laboratorijih za generiranje kvalitetnih setov podatkov.

Ključne besede: mikrobiologija / sesalci / prebavni trakt / mikrobiota / metabolno profiliranje / študentsko delo / kakovost

1 INTRODUCTION

The status of intestinal microbiota (IM) has been shown to be decisively linked to host well-being (Jin *et al.*, 2012) through direct contact with its immune system, the identity, rates and periodicity of conversion of organic compounds to various metabolites, all of which were to distinct extent linked to numerous chronic alterations on intestines (Marchesi, 2007). IM thus plays crucial role in host absorption of organic components, development and well-being of healthy intestinal tract but also its immune system related to resistance and/or resilience towards invading and pathogenic species (Jacobs *et al.*, 2009; Jin *et al.*, 2012).

Despite the fact that many environmental factors influence microbial community structure, activity and the surrounding environmental parameters of intestinal tract depending on the location within it, feed, and health, it has become apparent that microbial function and structure are relatively stable, providing the basis for energy conversion and stable coexistence of various hosts with their specific intestinal microbiota (Turnbaugh *et al.*, 2009; Jin *et al.*, 2012) through geological time-scales.

As methodological principles have been implicated as sources of major systematic bias when comparing microbial communities between groups of organisms of the same species or breed and especially between different species (Lin *et al.*, 1997; Simpson *et al.*, 2002; Ley *et al.*, 2008), an attempt was made to set-up and test a number of methods on contrasting samples from distinct species in order to identify the feasibility of such joint and all-inclusive approach across lineages of hosts. In this study, we focused on setting up four groups of methods in order to provide a standardized basis for future routine comparisons of distinct microbiomes from contrasting samples: (i) analysis of immediate physical-chemical parameters, (ii) microbial metabolic capacity of attached and planktonic cells in relation to intact community, (iii) microbial community structure of bacteria and archaea, (iv) analytical approaches for data analysis and integration. Four mammalian species were selected as sources of intestinal tract samples as model samples covering the large extent of differences between types of intestinal tracts (Simpson *et al.*, 2002): herbivores (ruminants (prepeptic fermentation), rodent (postpeptic fermentation), carnivores and omnivores.

The second aim of this study was to assess the competence of a group of students to perform a specified range of tests and measurements during their Master of Science studies, to evaluate the resulting spread in collected data and to foster awareness of the relevance and importance of their activities simulating a regular laboratory work day and respective duties.

2 MATERIALS AND METHODS

2.1 SAMPLING AND PHYSICAL-CHEMICAL ANALYSES

The following species were selected for analyses: rabbit (*Oryctolagus cuniculus*), cow (*Bos taurus*), swine (*Sus scrofa*) and dog (*Canis lupus familiaris*). Fresh stool samples were collected aseptically in polyvinyl bags and stored at 4 °C during transport to laboratory. Dry matter, organic matter and pH were determined according to APHA 2005. Dry matter of sample aliquots was determined by overnight drying of cups (Staatlich KPM, Berlin, Germany) in Instrumentaria ST-05 (Instrumentaria, Croatia) at 105 °C, whereas organic matter was determined by weight-loss on combustion at 550 °C. pH was determined in 10 g aliquots amended with 25 mL dH_2O using Orion 520A (Thermo Fisher Scientific, Inc. USA). Molecular weight index of water soluble organic compounds (MWI) was determined in 1 mL aliquots of sample suspensions obtained in pH measurements**.** After 2 min centrifugation at 13.000 rpm supernatant spectral properties (200–800 nm) were determined in 500 uL quartz kivette Hellma 150-QS (Hellma, Germany) using spectrophotometer Shimadzu UV-160A (Shimadzu, Japan) (Twardowsky *et al.*, 2004). MWI was calculated as a ratio of A365 nm to A250 nm. Concentration of soluble organic carbon in samples was determined in the form of chemical oxygen demand in aliquots derived from the same supernatant as MWI according to Clescerl *et*

al. (1999). After centrifugation at 10.000 rpm for 5 min 33 µL of sample were mixed with 670 µL of reagent and 297 µL of dH2O and incubated for 2h at 150 °C. Samples were cooled, centrifuged for 10 min at 3000 rpm and 200 µL aliquots transferred to microtiter plates for measurements at 595 nm using microtiter plate reader BIOTEK ELx808I. Standard curves for COD were prepared from glucose (Sigma) at concentrations of 0, 100, 300, 500, 700, 1000 µg/mL. Concentration of reducing sugars in samples was determined according to Lever (1977). Reagent aliquots of 1 mL were transferred to eppendorf tubes and amended with 20 µL of samples from centrifugation in COD determination and incubated at 100 °C for 10 min and 200 µL aliquots transferred to microtiter plates for measurements at 415 nm using microtiter plate reader BIOTEK ELx808I. Standard curves for reducing sugars were prepared from glucose (Sigma) at concentrations of 0, 100, 300, 500, 700, 1000 µg/mL. Total nitrogen was determined in modified Berthelot reaction using SAN plus Continuous Flow Analyzer according to manufacturer's instructions (Skalar Analytical B.V., Netherlands) and calculated per gram of dry weight. Total inorganic carbon and volatile fatty acids (VFA) contents were determined in as described before (Kolbl *et al.*, 2014).

2.2 MICROBIOTA FRACTIONATION AND METABOLIC PROFILING

Aliqots of samples (10 g) were amended with anaerobic phosphate-buffer, homogenized by shaking for 20 min at 350 rpm. Planctonic fraction from this homogenate was recovered by sieving (#150 um), washed by anaerobic buffer to produce 350 mL and made completely anaerobic by purging with N2. The material retained on sieve was again transferred to phosphate buffer with 0.001 % CTAB and homogenized by shaking for 20 min at 350 rpm after which the sieving procedure was repeated to produce 350 mL of the attached fraction. Sample aliquots of 10 g were transferred to 350 mL of phosphate-buffer to produce the total community suspension. Five distinct substrates (yeast extract, starch, pectin, xylan, peptone) were used in metabolic profiling of the three fractions at 20 g/L. Preheated substrate aliquots of 45 mL were amended with 10 mL of each of three fractions in separate falcon tubes and incubated at 38 °C for three hours. Negative controls were not amended with organic substrates. The concentrations of five short chain volatile fatty acids and ethanol were determined in sample headspace (Kapr, Val, Prop, But, Oc) using gas chromatography. The relative VFA profiles were analyzed.

2.3 PROFILING OF BACTERIAL AND ARCHAEAL COMMUNITY STRUCTURE

Microbial community DNA was extracted from 0.5 g aliquots using »PowerSoil® DNA Isolation Kit« according to manufacturer's instructions. Nanovue was used to determine the quality and purity of extracted DNA. Two pairs of primers were used in PCR targeting genes for 16S rRNA: 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and U518r (5'-ATTACCGCGGCTGCTGG-3') for bacteria; 109f (5'-ACNGCTCAGTAACRCGYR-3') and U518r for archaea using the conditions described before (Stres *et al.*, 2008; Zajec *et al.*, 2012). PCR products were purified using High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and separate restriction reactions were prepared for *HhaI* and *MspI* endonucleases as described before (Novak *et al.*, 2013). After separation of labelled fragments in ABI 3130*xl*GeneticAnalyzer (AppliedBiosystems, Carlsbad, USA).

2.4 STATISTICAL ANALYSIS

Results from physical-chemical analyses and metabolic profiling were log-transformed and analysed in PAST (Hammer *et al.*, 1999) using non-metric multidimensional scaling and NP-MANOVA. BioNumerics software (Applied Maths NV, 2011) was used for analyses of molecular dataset using Pearson's correlation coefficient and Nearest Neighbor Joining taking into account only fragments representing 0.5 % of the total profile, as described before (Stres *et al.*, 2008).

3 RESULTS AND DISCUSSION

The differences in physical-chemical parameters in collected samples were effectively recovered (Table 1) and enabled clear separation of samples according to animal source (Fig. 1). From the perspective of the analytical approaches it is worth noticing that the lowest concentration of reducing sugars were detected in fecal samples of pig, whereas highest in samples of dog and rabbit. On the other hand, the ratio of VOA/TIC was highest in bovine samples and lowest in those of pig. This shows that the adopted approach was successful in mapping concentration of chemical parameters that have been associated with a number of diseases in medicine and veterinary medicine (Gerritsen *et al.*, 2011; Tlaskalová-Hogenová *et al.*, 2011). In addition, it clearly showed that the four types of animals produced significantly different sets of habitats for microbial communities in line with the general differences in characteristics of intestinal tracts.

Table 1: *Physical-chemical characteristics of fecal samples (± standard deviation)*
Preglednica 1: *Fizikalno-kemijske lastnosti vzorcev posameznih živali (± standardni odklon)*

Properties	rabbit	dog	cow	pig
pH	7.59 ± 0.05	6.70 ± 0.01	7.21 ± 0.1	6.94 ± 0.02
% DM	56.30 ± 1.66	28.42 ± 0.00	13.07 ± 0.09	26.41 ± 0.00
% OSOM/g DM	88.57 ± 1.27	83.33 ± 0.00	83.99 ± 0.52	88.24 ± 0.00
% N_{tot} in DM	1.26 ± 0.00	0.95 ± 0.00	0.18 ± 0.00	0.55 ± 0.00
OC (mg/g SSDM)	502.37 ± 96.64	881.11 ± 38.08	1284.20 ± 195.15	876.25 ± 45.13
MWI	0.50 ± 0.00	0.49 ± 0.00	0.49 ± 0.01	0.48 ± 0.01
% DM 150 µum	22.86	10.00	9.16	8.13
% OM/ g DM 150 µum	91.67	88.24	92.31	92.86
VOA (mg/L)	2968 ± 131	5981 ± 151	1766 ± 98	1541 ± 101
TIC (mg/L)	1431 ± 77	3973 ± 105	692 ± 83	1284 ± 97
VOA/TIC	2.07	1.51	2.55	1.20

Analyses of bacterial and archaeal microbial communities revealed that the adopted approach of fast fingerprinting was effective in delineating microbial communities present in different intestinal habitats of four mammalian species. Results showed that the four habitats were characterized by markedly different bacterial and archaeal microbial communities, in line with the observation of the differences in the physical-chemical characteristics of the collected samples.

Results of metabolic profiling of residing microbial communities were in contrast to the results of physical-chemical analyses and microbial community structure in bacterial and archaeal microbial communities. Fig. 3 shows that metabolic profiles of microbial communities in response to 5 different polymeric substrates were largely overlapping. This shows that microbial communities resident in significantly different environments and of distinct assembly can have largely similar functional

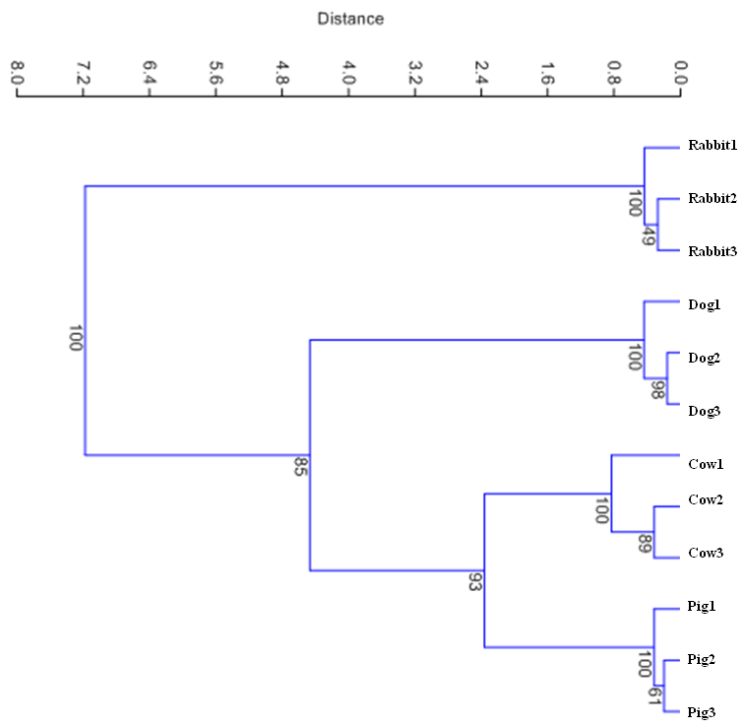

Figure 1: *Dendrogram showing similarities in physical-chemical characteristics of animal samples*
Slika 1: *Dendrogram podobnosti fizikalno-kemijskih lastnosti fecesa proučevanih živali*

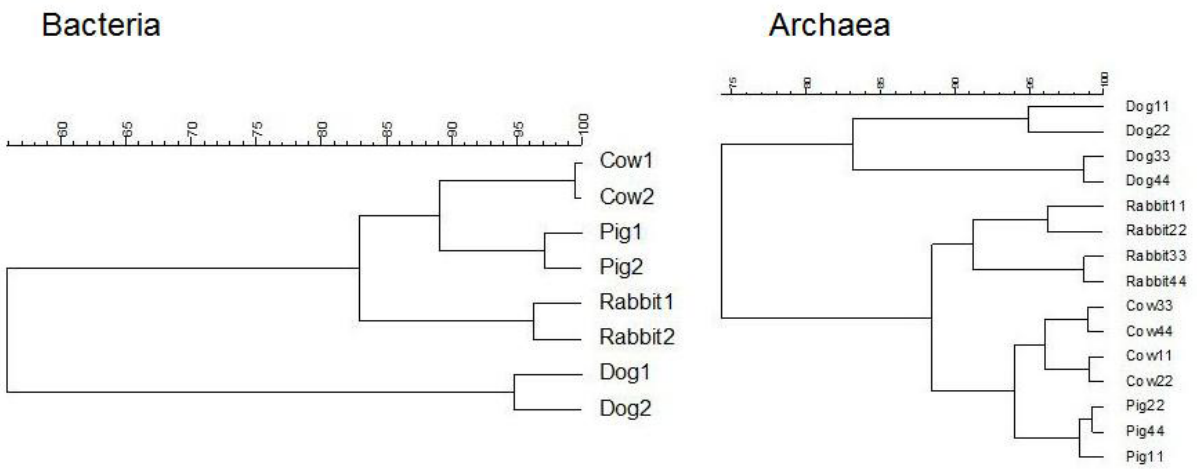

Figure 2: *Dendrogram showing similarities of bacterial (left) and archaeal (right) microbial communities in samples of dog, cow, rabbit and pig.*
Slika 2: *Dendrogram podobnosti bakterijskih združb v fecesu psa (Dog1, Dog2), goveda (Cow1, Cow2), kunca (Rabbit1, Rabbit2) in prašiča (Pig1, Pig2). Desno: dendrogram podobnosti arhejskih združb v fecesu psa (Dog11, Dog22, Dog33, Dog44), goveda (Cow11, Cow22, Cow33, Cow44), kunca (Rabbit11, Rabbit22, Rabbit33, Rabbit44) in prašiča (Pig11, Pig22, Pig33, Pig44)*

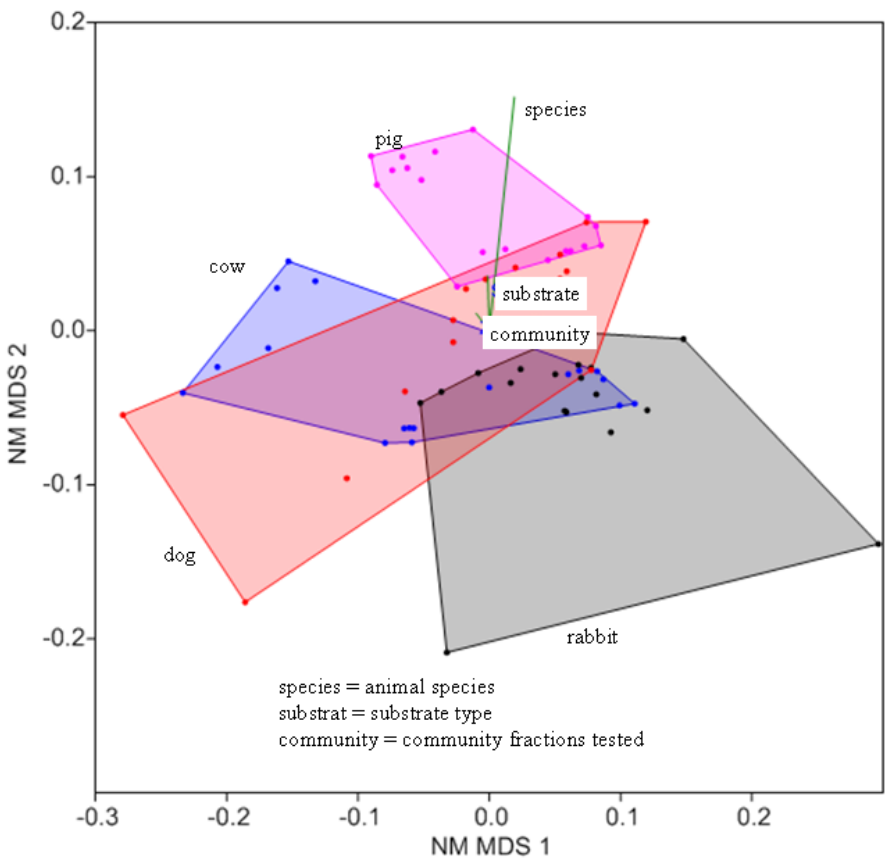

Figure 3: *Nonmetric multidimensional scaling (NM-MDS) ordination of physiological responses of fractions of microbial communities based on Bray-Curtis similarity. (Stress= 0.059)*
Slika 3: *Nemetrično večdimenzionalno lestvičenje (NM-MDS) fizioloških odzivov različnih frakcij mikrobnih združb, izrisano na podlagi Bray-Curtisovega indeksa podobnosti. Kunec (rabit), govedo (cow), prašič (pig), pes (dog), testne frakcije združbe (community). (Stres= 0,059)*

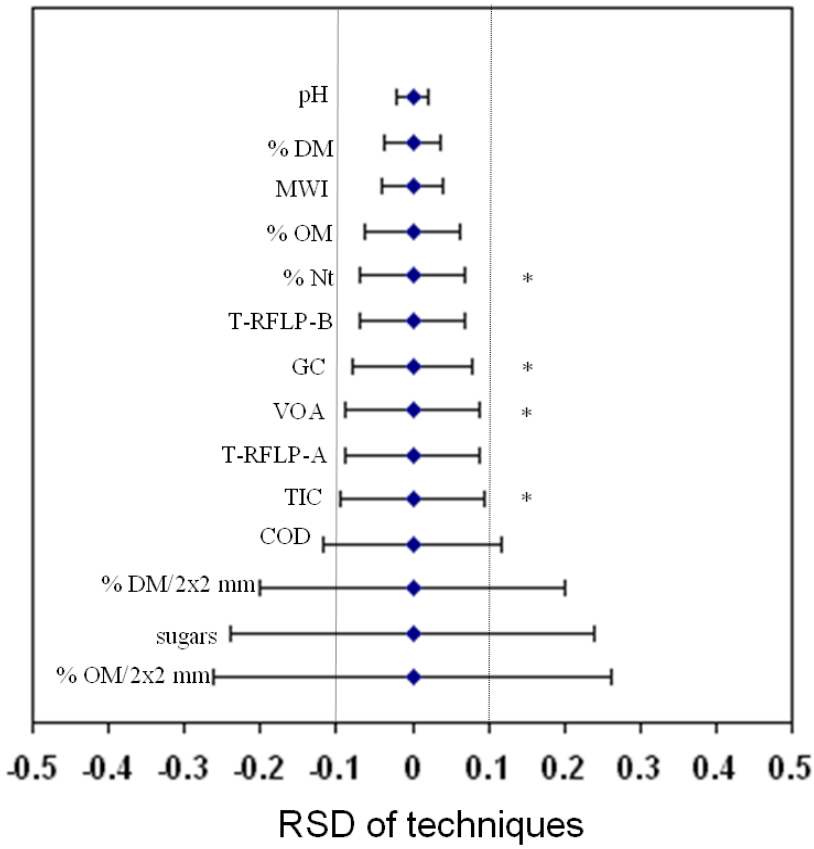

Figure 4: *Relative standard deviation of techniques used by students in comparison to fully automated approaches*
Slika 4: *Relative standardni odklon tehnik, ki so jih uporabili študenti v primerjavi z avtomatiziranimi pristopi*

potential. Fractionation of microbial communities to total, particle-bound and particle-free fractions did not result in significant differences between responses, indicating that functionally similar microbial communities were present in the three fractions, despite differences in lifestyle (adherence). The largest portion of variance present in metabolic profiles was represented by the animal type, the second, and four times less important by the choice of substrates used in experiment, whereas the fractionation was of least importance, in-line with above observations.

As different types of intestinal tracts are characterized by significant differences in anatomy, function, length, and also significant differences have been observed in microbial communities, their traits and functional capabilities, it would be inappropriate to perform comparisons of distinct sections of intestinal tracts between different mammalian species. In addition, direct sampling of intestinal contents represents an additional and measurable portion of stress for test animals and should be avoided. Consequently, faecal samples were selected as most acceptable samples for high-throughput analyses of microbial characteristics, functions and environmental characteristics especially as they represent an accepted venue to infer animal and human health problems.

From ecological point of view, the results showed that different environments present in the last part of contrasting intestinal tracts of four mammalian species represent ecologically different niches that contained microbial communities of distinct assembly, but of overlapping metabolic potential.

Finally, the results suggest (i) that the operators independently organized and shared tasks;(ii) successfully completed all methods;(iii) obtained relevant information;(iv) critically evaluated and interpreted the data within the extent of their knowledge; (v) that relative standard deviation(RSD) typically could be compared to those of the automated analytical procedures(RSD < 10 %) (Fig. 4) and therefore represented the maximum extent of the variability of the biological material itself. It follows that the motivated MSc students were able to uphold the unknown protocols under supervision and perform laboratory and analytical complex experimental task, process and interpret results, and approximate performance of analytical procedures

in industrial laboratories to generate data sets of acceptable high-quality.

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5 REFERENCES

Clescerl L.S., Greenberg A.E., Eaton A.D. 1999. Standard Methods for Examination of Water & Wastewater. 20th edition. Washington, DC: American Public Health Association.

De Graaf A.A., Maathuis A., de Waard P., Deutz N.E.P., Dijkema C. *et. al.* 2010. Profiling human gut bacterial metabolism and its kinetics using [U-13C] glucose and NMR. NMR Biomed., 23: 2–12, doi:10.1002/nbm.1418

Dumas M.E., Barton R.H., Toye A., Cloarec O., Blancher C., Rothwell A., Fearnside J., Tatoud R., Blanc V., Lindon J.C., Mitchell S.C., Holmes E., McCarthy M.I., Scott J., Gauguier D., Nicholson J.K. 2006. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc. Natl. Acad. Sci. USA, 103, 33: 12511–12516, doi:10.1073/pnas.0601056103

Gueimonde M., Tölkkö S., Korpimäki T., Salminen S. 2004. New Real-Time Quantitative PCR Procedure for Quantification of Bifidobacteria in Human Fecal Samples. Appl Environ Microbiol.: 4165–4169, doi:10.1128/AEM.70.7.4165-4169.2004

Hammer Ø., Harper D.A.T., Ryan P.D. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica, 4, 1: 9 p.

Holmes E., Li J.V., Athanasiou T., Ashrafian H., Nicholson J.K. 2011. Understanding the role of gut microbiome–host metabolic signal disruption in health and disease. *T*rends in Microbiology, 19, 7: 349–359, doi:10.1016/j.tim.2011.05.006

Jacobs D.M., Gaudier E., van Duynhoven J., Vaughan E.E. 2009. Non-digestible food ingredients, colonic microbiota and the impact on gut health and immunity: a role for metabolomics. Curr Drug Metab., 10, 1: 41–54, doi:10.2174/138920009787048383

Jin J.S., Touyama M., Kibe R., Tanaka Y., Benno Y., Kobayashi T., Shimakawa M., Maruo T., Toda T., Matsuda I., Tagami H., Matsumoto M., Seo G., Chonan O., Benno Y. 2012. Analysis of the human intestinal microbiota from 92 volunteers after ingestion of identical meals. Benef Microbes, 4, 2: 187–193, doi:10.3920/BM2012.0045

Kolbl S., Paloczi A., Panjan J., Stres B. 2014. Addressing case specific biogas plant tasks: Industry oriented methane yields derived from 5L Automatic Methane Potential Test Systems in batch or semi-continuous tests using realistic inocula, substrate particle sizes and organic loading. Bioresource technology, 153: 180–188, doi:10.1016/j.biortech.2013.12.010

Lever M. 1977. Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAHBAH): Effect of bismuth on the reaction. Analytical Biochemistry, 81: 21–27, doi:10.1016/0003-2697(77)90594-2

Ley R.E., Hamady M., Lozupone C., Turnbaugh P.J., Ramey R.R., Bircher J.S., Schlegel M.L., Tucker T.A., Schrenzel M.D., Knight R., Gordon J.I. 2008. Evolution of mammals and their gut microbes. Science, 320: 1647–1651, doi:10.1126/science.1155725

Li F., Hullar M.A.J., Lampe J.W. 2007. Optimization of terminal restriction fragment polymorphism (TRFLP) analysis of human gut microbiota. J Microbiol Methods, 68, 2: 303–311, doi:10.1016/j.mimet.2006.09.006

Lin C., Raskin L., Stahl D.A. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. FEMS Microbiol. Ecol., 22: 281–294, doi:10.1111/j.1574-6941.1997.tb00380.x

Lozupone C.A., Stombaugh J.I., Gordon J.I., Jansson J.K., Knight R. 2012. Diversity, stability and resilience of the human gut microbiota. Nature, 489, 7415: 220–230, doi:10.1038/nature11550

Marchesi J.R., Holmes E., Khan F., Kochhar S., Scanlan P., Shanahan F., Wilson I.D., Wang Y. 2007. Rapid and noninvasive metabonomic characterization of inflammatory boweldisease. J Proteome Res, 6: 546–551, doi:10.1021/pr060470d

Pieper R., Kröger S., Richter J. F., Wang J., Martin L., Bindelle J., Htoo J.K., Smolinski D., Vahjen W., Zentek J., Kessel A.G. 2012. Fermentable Fiber Ameliorates Fermentable Protein-Induced Changes in Microbial Ecology, but Not the Mucosal Response, in the Colon of Piglets. J Nutr., 142, 4: 661–66, doi:10.3945/jn.111.156190

Simpson J.M., Kocherginskaya S.A., Aminov R.I., Skerlos L.T., Bradley T.M., Mackie R.I. *et al.* 2002. Comparative microbial diversity in the gastrointestinal tracts of food animal species. Integr Comp Biol, 42: 327–331, doi:10.1093/icb/42.2.327

Stres B., Danevcic T., Pal L. *et al.* 2008. Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. FEMS Microbiol Ecol. 66: 110–122, doi:10.1111/j.1574-6941.2008.00555.x

Turnbaugh P.J., Hamady M., Yatsunenko T., Cantarel B.L., Duncan A., Ley R.E., Sogin M.L. *et al.* 2009. A core gut microbiome in obese and lean twins. Nature, 457: 480–484, doi:10.1038/nature07540

Tlaskalová-Hogenová H., Stěpánková R., Kozáková H., Hudcovic T., Vannucci L., Tučková L., Rossmann P., Hrnčíř T., Kverka M., Zákostelská Z., Klimešová K., Přibylová J., Bártová J., Sanchez D., Fundová P., Borovská D., Srůtková D., Zídek Z., Schwarzer M., Drastich P., Funda D.P. 2011.

The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol, 8: 110–120, doi:10.1038/cmi.2010.67

Tims S., Zoetendal E.G., de Vos W.M., Kleerebezem M. 2010. Chapter 2: Host Genotype and the Effect on Microbial Communities. In: Metagenomics of the Human Body. Nelson K.E. (ed.). Springer: 15–41

TNO. 2013. Gastrointestinal models (TIM) with high predictive power. Netherlands Organisation for Applied Scientific Research TNO. http://www.tno.nl/content.cfm?context=thema&content=markt_product&laag1=891&laag2=195&laag3=320&item_id=1100&Taal=2 (20. Oct. 2013)

Twardowski M.S., Boss E., Sullivan J.M., Donaghay P.L. 2004. Modeling the spectral shape of absorption by chromophoric dissolved organic matter. Marine Chemistry, 89, 1–4: 69–88, doi:10.1016/j.marchem.2004.02.008

Zajec N., Stres B., Avguštin G. 2012. Distinct approaches for the detection and removal of chimeric 16S rRNA sequences can significantly affect the outcome of between-site comparisons. Aquat. Microb. Ecol., 66: 13–21, doi:10.3354/ame01510