GOBIERNO de CANTABRIA

Consejería de Desarrollo Rural, Ganadería, Pesca y Alimentación.
Dirección General de Desarrollo Rural.

UC UNIVERSIDAD DE CANTABRIA

CENTRO DE INVESTIGACIÓN Y FORMACIÓN AGRARIAS CIFA

Animal Production System Area

The characterization of the microbiome and resistome in feces and slurry of dairy cows in different livestock production systems

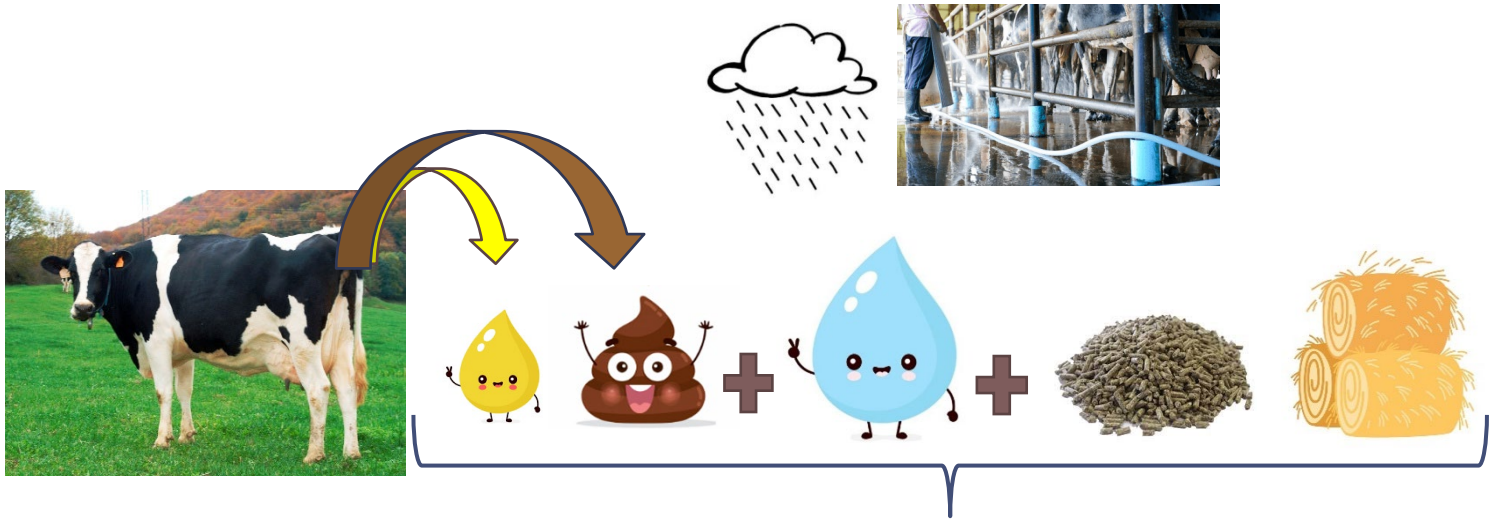
Nejjam I. and Varsaki A.

September, 01st 2024 (Florence, Italy)

Cantabria, Spain

Figure 1: The 17 autonomous regions of Spain (http://mapasenpdf.com/descarga?pdf=maps%2Feducational%2Fespana%2Fpdfs%2Fmapa-politico-espana-para-imprimir.pdf)

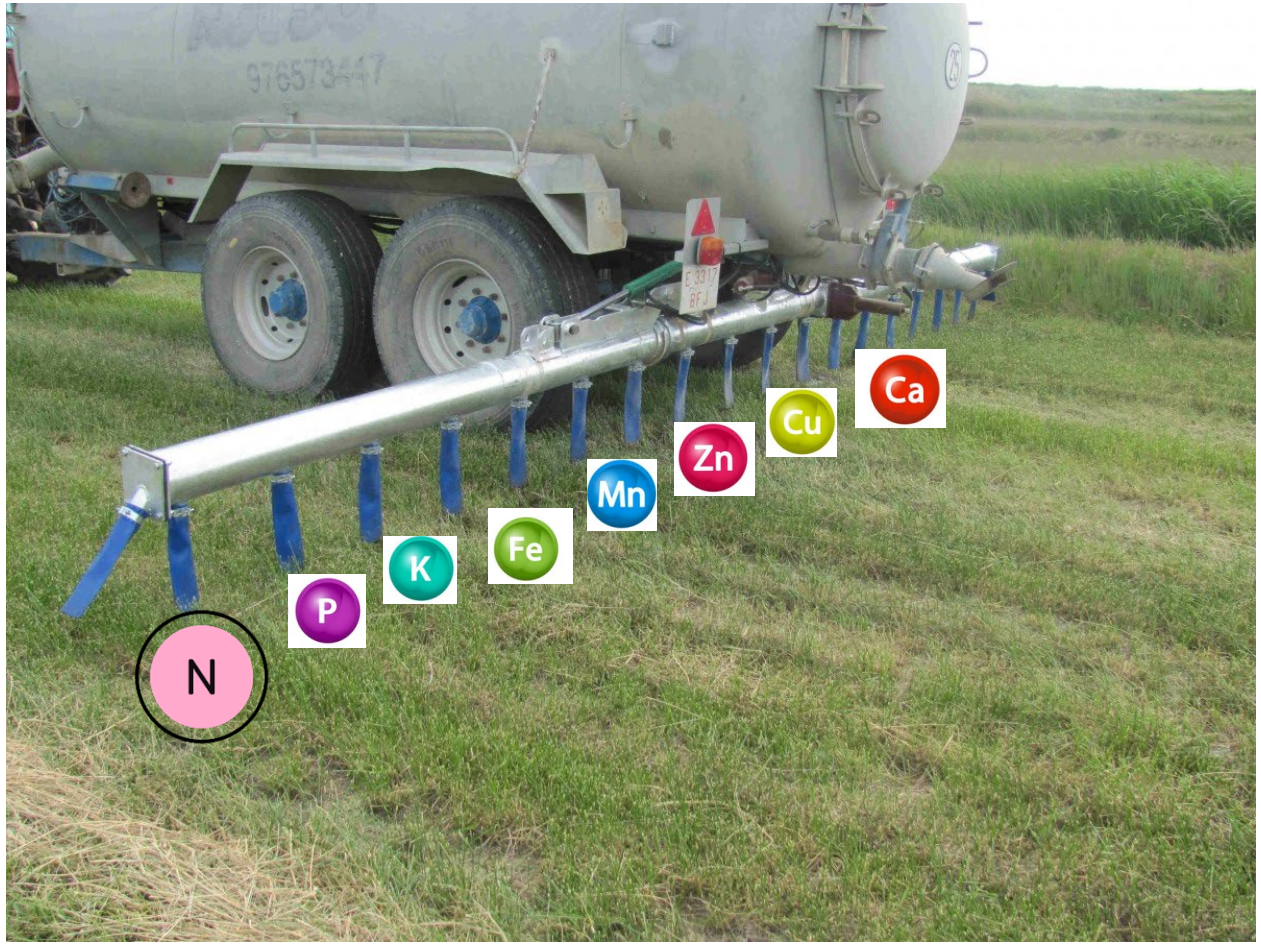
- Heads of dairy cattle: **164,406** (MAFF, 2024)
- Number of dairy cattle farms: **838** (MAFF, 2024)



Slurry production

55 l/cow/day → 9 000 tons

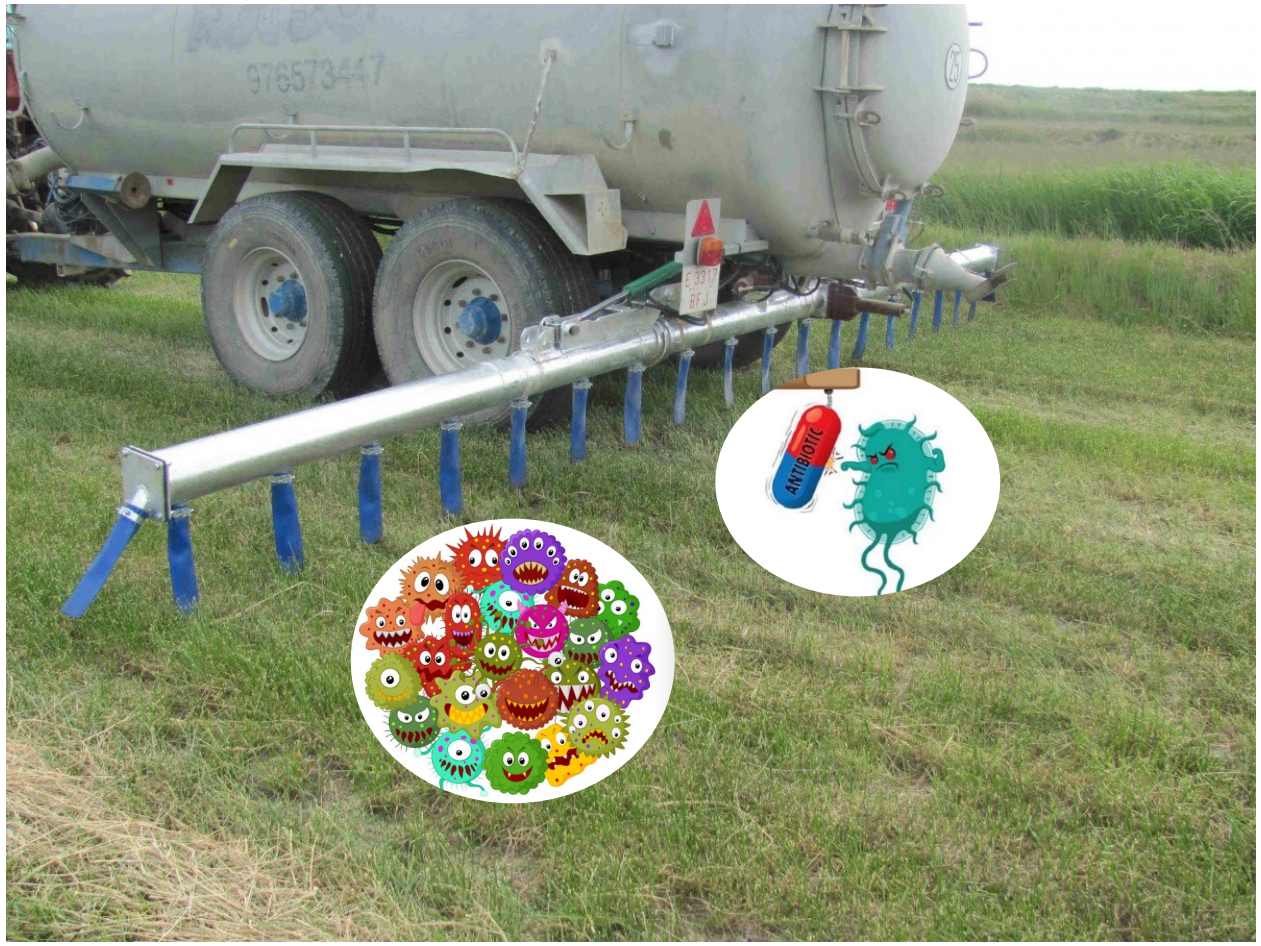
Used as an organo-mineral fertilizer



- High agronomic value;

➢ Soil quality improvement

➢ Yield and quality improvement of crop and pasture



- Pathogenic and non-pathogenic bacteria
- Bacteria and antimicrobial resistance genes

World Health Organization

By **2050**, multi-resistant bacteria could kill **10 million people** per year, surpassing cancer as our main health concern (WHO, 2021).

Hypothesis

H_0 : Livestock farms present the same ARG and ARB load according to the production system, and there are no significant differences.

H_a : Livestock farms present different ARG and ARB load according to the production system, and there are significant differences.

Microbiome

Resistome

Intensive system

Conventional grazing system

Organic grazing system

During 4 years (2021-2024)

1- Selection of production systems and farms

Livestock Production Systems

Conventional grazing

Organic grazing

Intensive

2- Questionnaires realization

3- Cows selection

Farm × 6 × 100 × 30

Farm × 6 × 100 × 30

Farm × 6 × 100 × 30

first-calving Holstein cows

under the official milk recording

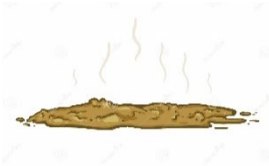
without AB treatment in the last 6 months

Total feces samples = 300

Total slurry samples = 90

4- Sampling

Collection from the rectum



Collection from piles of accumulated slurry

During 4 years (2021-2024)

1- Selection of livestock production systems and farms

Livestock Production Systems

Conventional grazing

Organic grazing

Intensive

2- Questionnaire realization

Farm × 6

Farm × 6

Farm × 6

3- Cows selection

first-calving Holstein cows

under the official milk recording

without AB treatment in the last 6 months

× 100

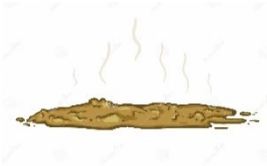
× 30

les (final objective) = **300**

les (final objective) = **90**

4- Sampling

Collection from the rectum



Collection from piles of accumulated slurry

Physicochemical conditions?

Multiparameter (HI 991301, Hannah Instruments)

Physicochemical analysis

Physical parameters

- pH
- Electrical conductivity
- Total dissolved solids (TDS)

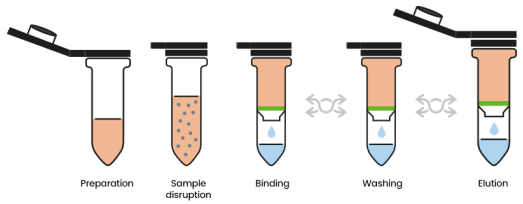
Chemical parameters

- Dry Matter(drying oven at 103°C)
- Ash (muffle furnace at 500°C)
- Cations (atomic absorption)
- Organic nitrogen (Kjeldahl method)
- Ammoniacal nitrogen (distillation)

Microorganisms ?

1- DNA extraction and purification

QIAmp PowerFecal Pro DNA Kit



2- DNA quantification

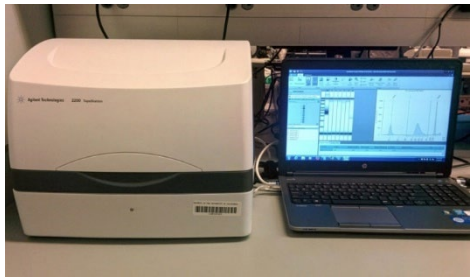
Estimate the concentration of extracted DNA by **fluorescence** using a fluorometer (Qubit 4.0)

3. DNA purity control

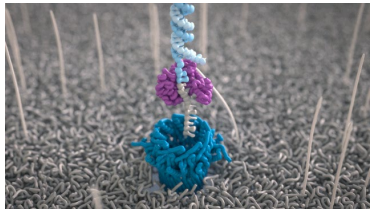
Verify the purity of DNA and the presence of other components (proteins, salts, etc.) using a spectrophotometer (V-730, Jascon) with 260/280 and 260/230 nm ratios

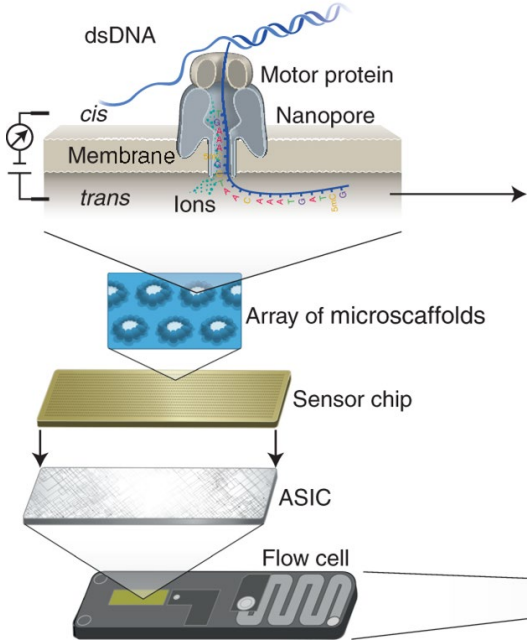
4. DNA quality control

Assess the degree of integrity using automated electrophoresis (Agilent TapeStation) to determine the DNA integrity number (**DIN**)

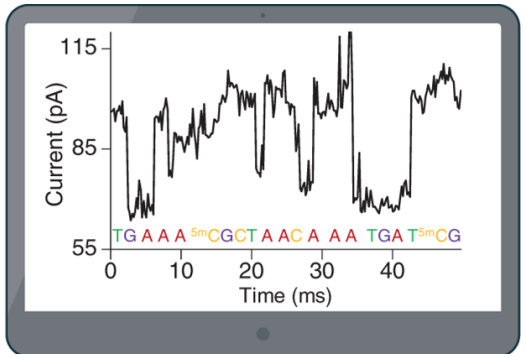


5- Library construction and sequencing



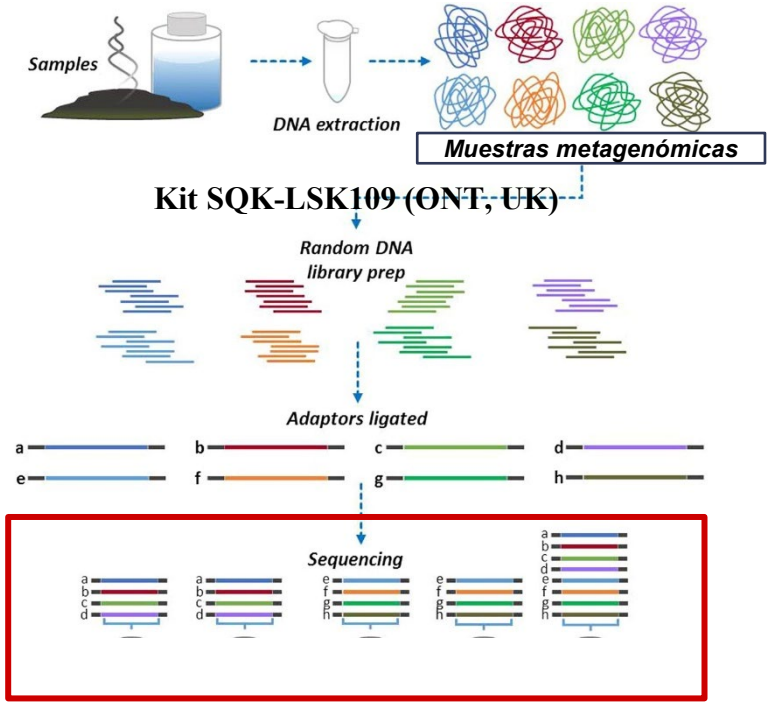


Basecalling (Guppy, SUP)



Fastq

Flow Cell



MinIon Mk1C device (Oxford Nanopore Technology)

6- Bioinformatic analysis

Metagenomics pipeline: SqueezeMeta (Tamames and Puente-Sánchez, 2019)

A fully automatic pipeline for metagenomics, covering all steps of the analysis: assembly, ORF prediction, annotation and binning.

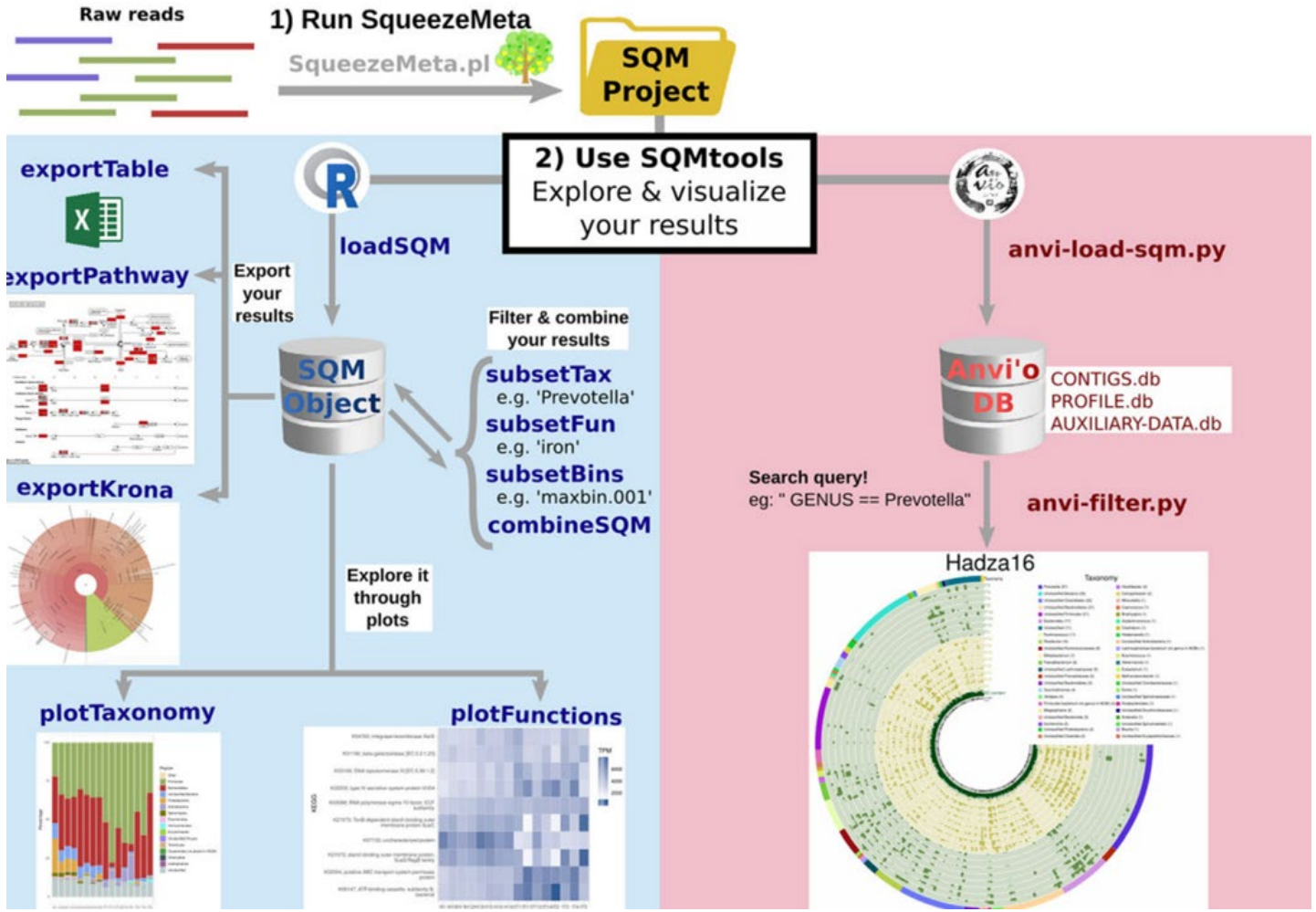


Figure2. Main workflow of the SQMtools pipeline(Tamames and Puente-Sánchez, 2019)

- Metagenomics pipeline: SqueezeMeta (Tamames and Puente-Sánchez, 2019)
- High Performance Computing (HPC) of Castilla and León (SCAYLE.

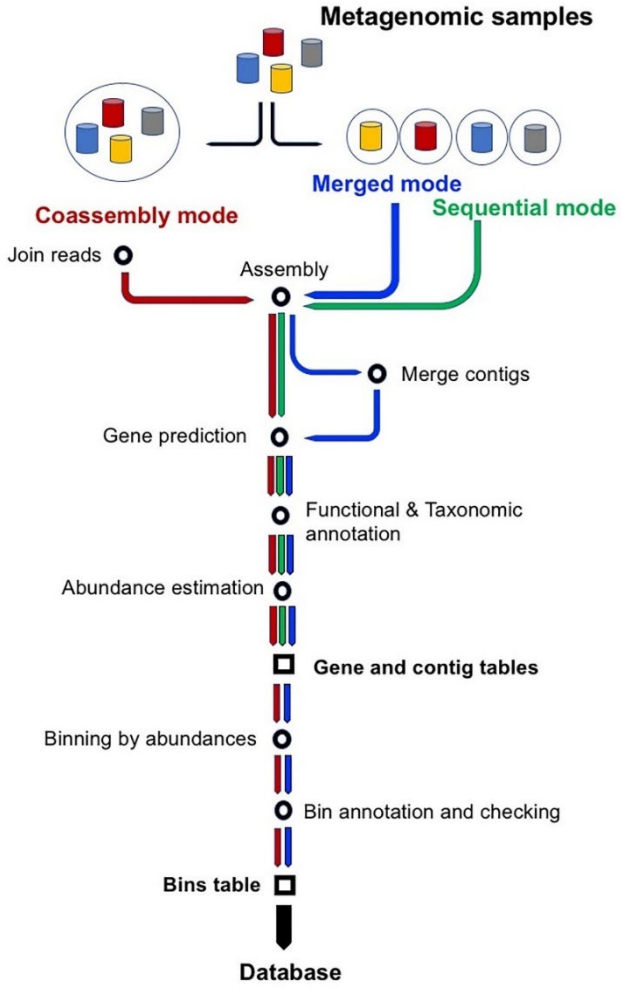
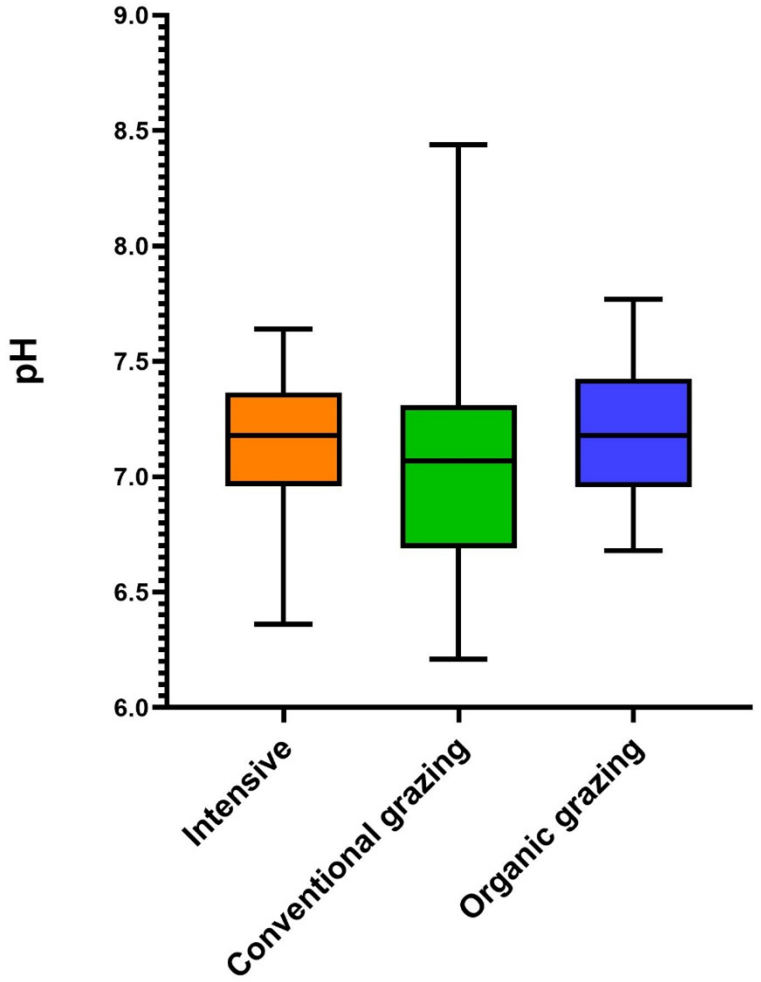
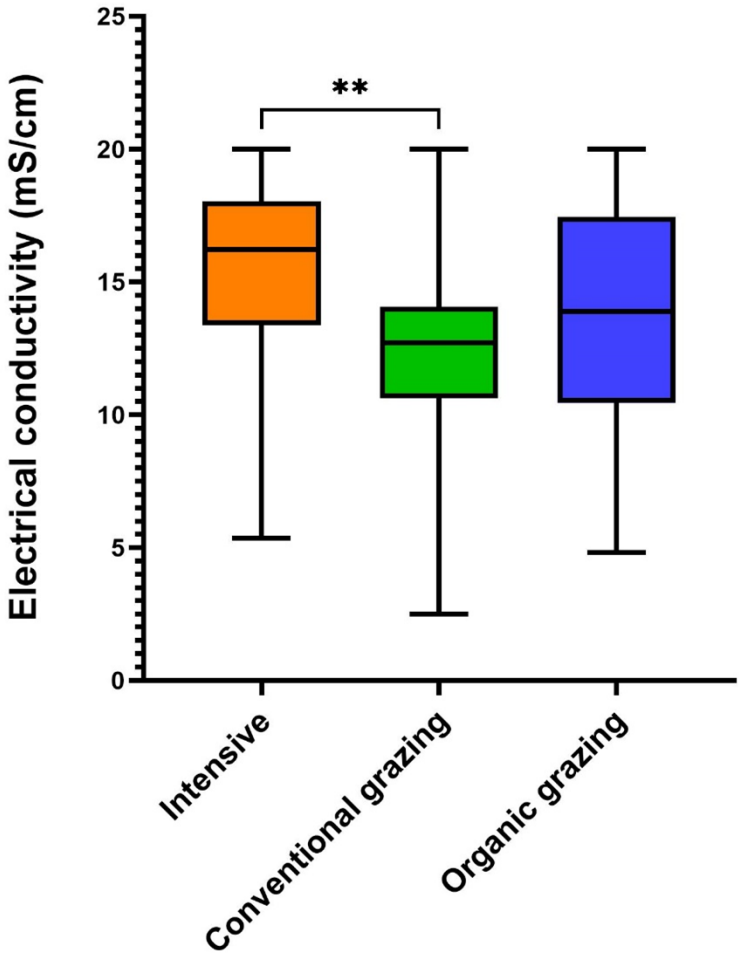
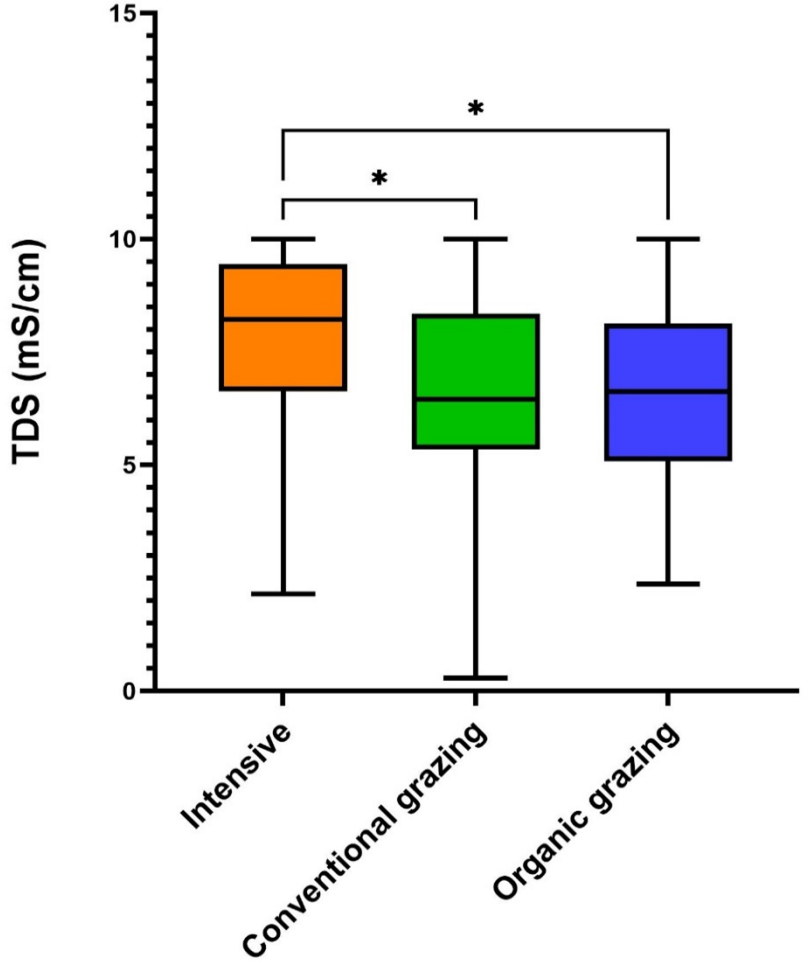


Figure 3. Workflow of the three modes of operation of SqueezeMeta: sequential, co-assembly and merged (Tamames and Puente-Sánchez, 2019)

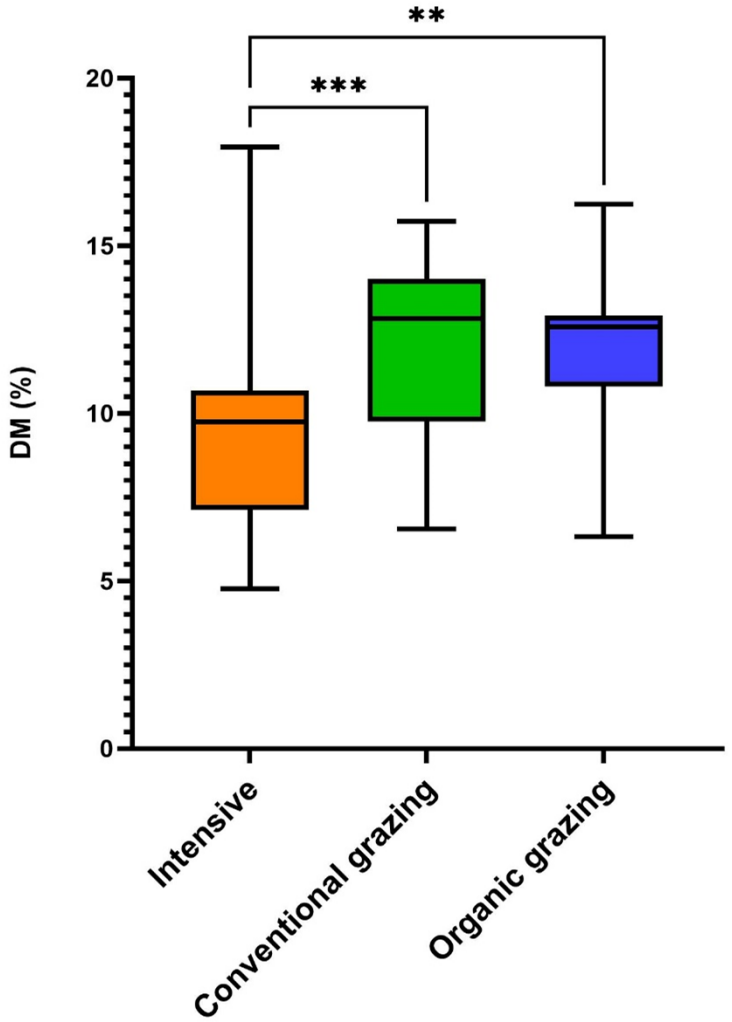


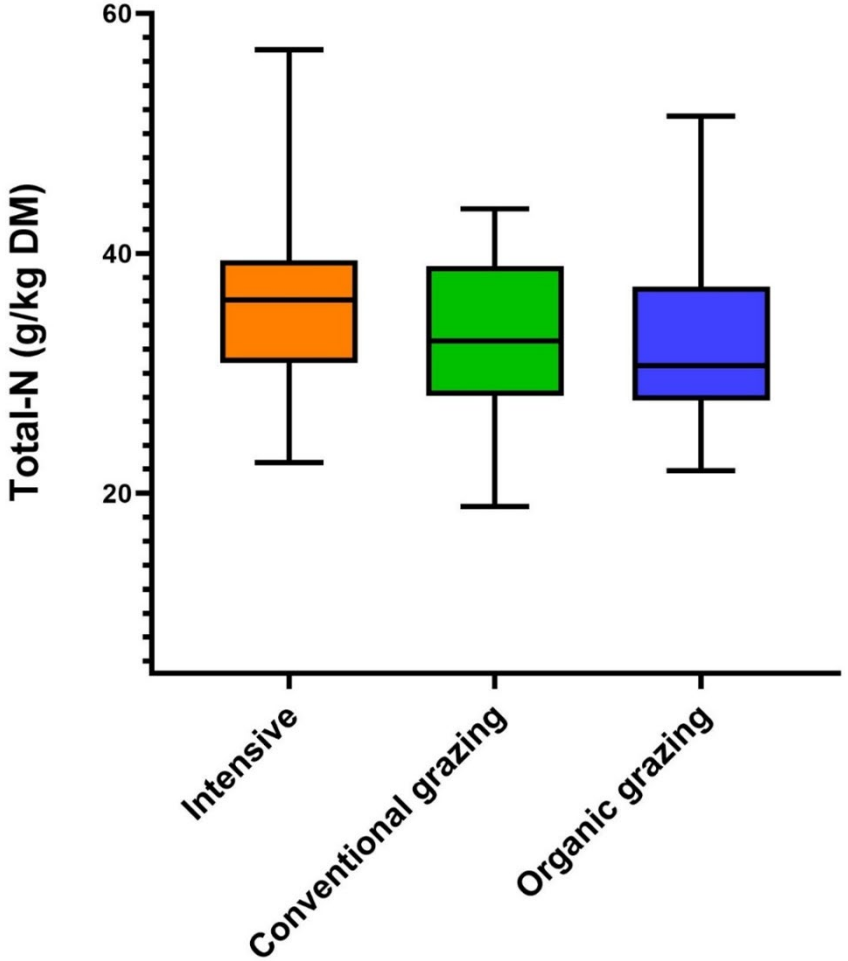


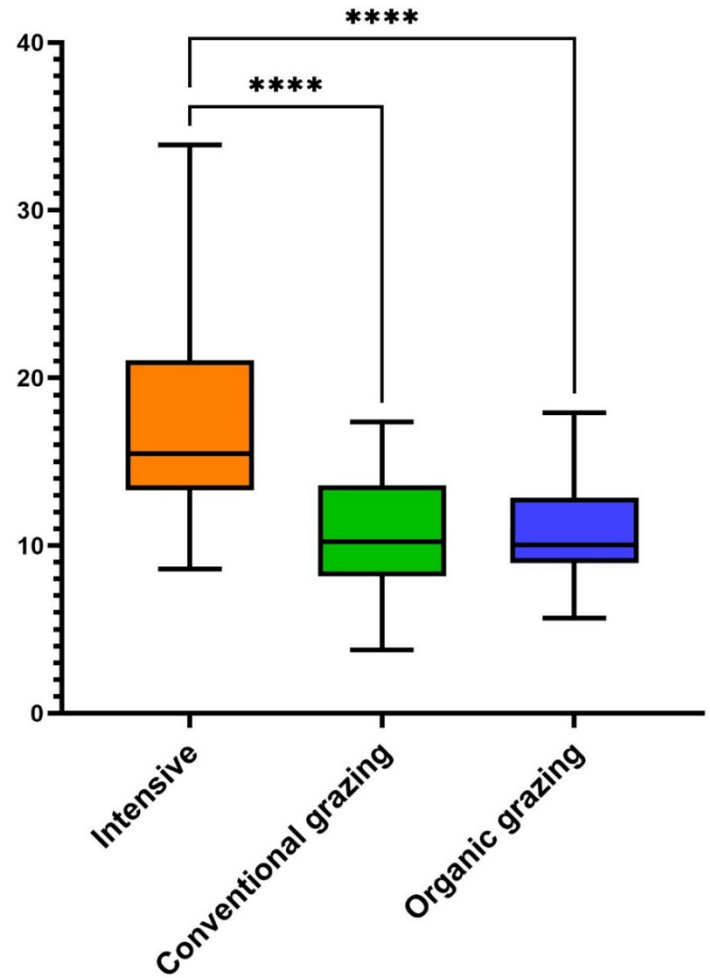


Intensive; N= 38
Conventional grazing; N= 33
Organic grazing; N= 30

*** p<0,05**
**** p<0,01**

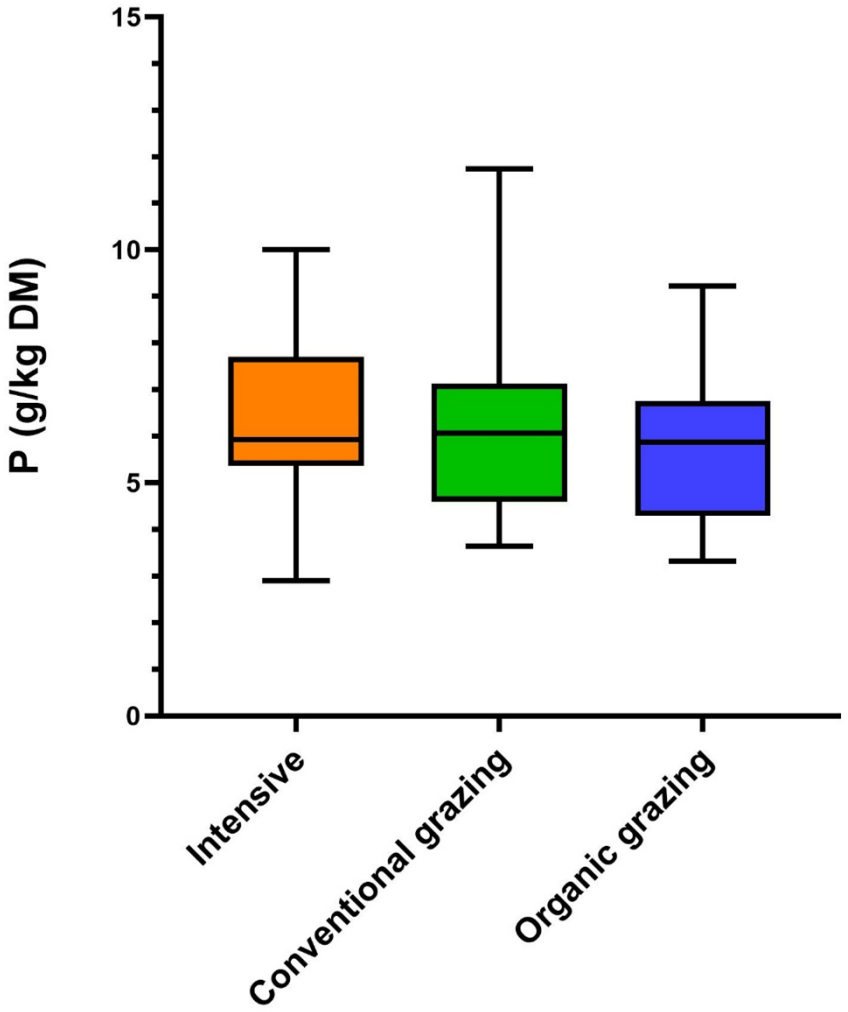


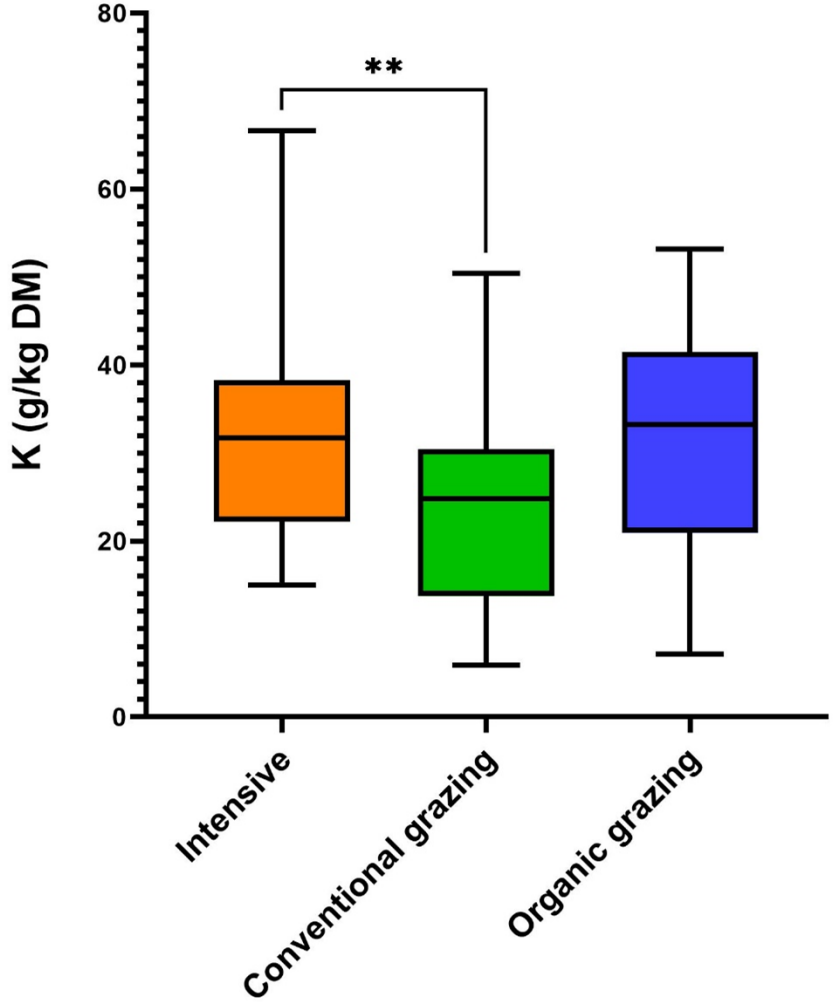




Intensive; N= 38
Conventional grazing; N= 33
Organic grazing; N= 30

** p<0,01
*** p<0,001
****p<0,0001





Intensive; N= 38
Conventional grazing; N= 33
Organic grazing; N= 30

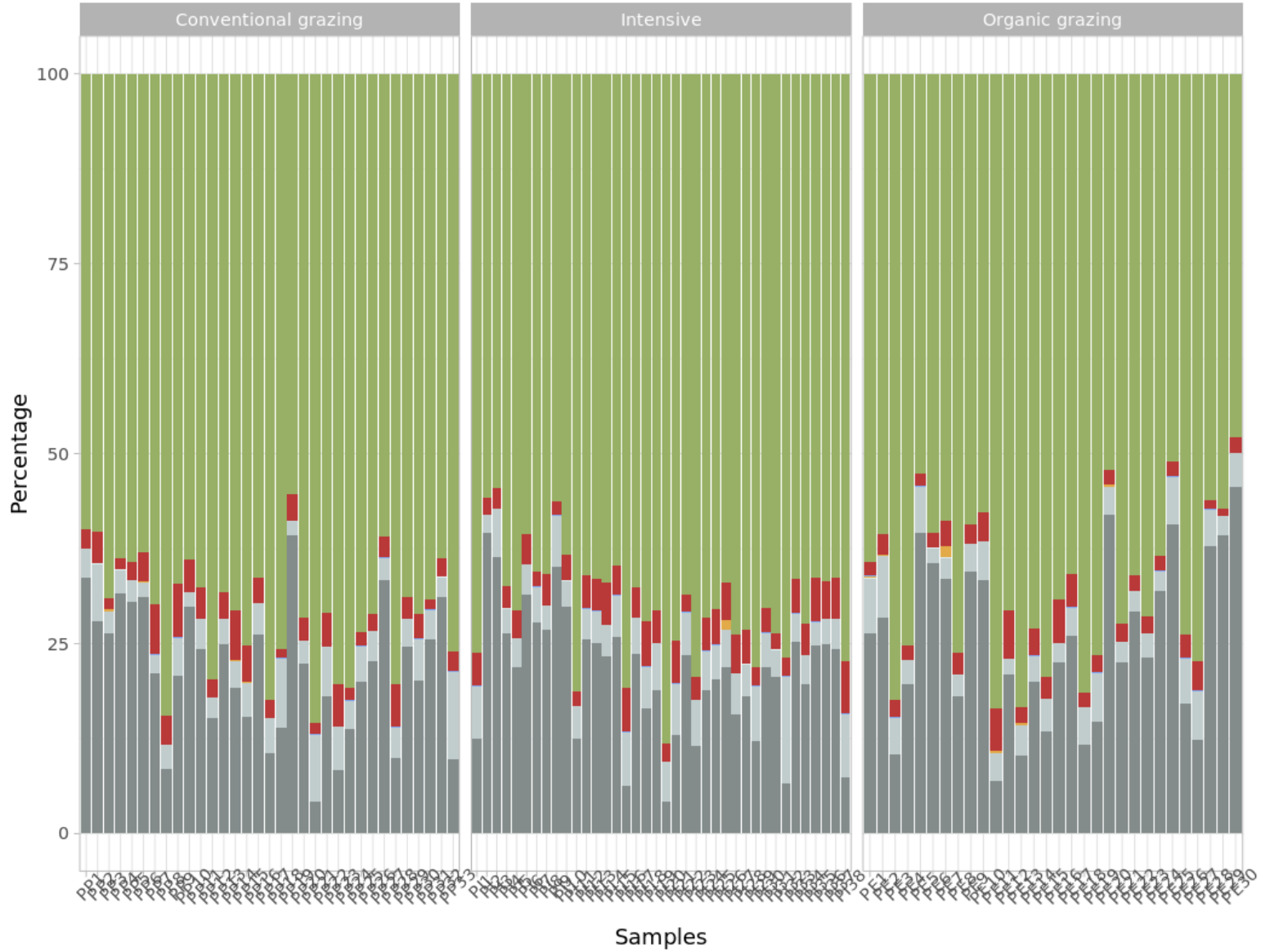
** p<0,01

Sequences characteristics of all samples (N=101)

Parameters	Average	Standard deviation
Mean read length (bp)	2,797	1,061
Mean read quality	13.31	0.21
Median read length (bp)	1,252	844
Median read quality of reads	14.30	0.27
Mean number of reads	319,894	298,769
Mean read length N50 (bp)	6,792	1,089
Mean of total bases (Gb)	7,80	6,80

- Long reads with a mean length of 2,797 bp and a median read quality >13
- Super accuracy basecalling mode (guppy version 6.4.8)

#---------- Statistics on contigs
Assembly
Number of contigs 276,472
Total length 4,364,454,890
Longest contig 1,431,698
Shortest contig 200
N50 29,031
N90 7,323
Contigs at superkingdom (k) rank 247,395 (89.5%), in 4 superkingdoms
Contigs at phylum (p) rank 221,583 (80.1%), in 76 phyla
Contigs at class (c) rank 186,684 (67.5%), in 81 classes
Contigs at order (o) rank 138,371 (50.0%), in 135 orders
Contigs at family (f) rank 78,734 (28.5%), in 238 families
Contigs at genus (g) rank 35,193 (12.7%), in 531 genera
Contigs at species (s) rank 26,439 (9.6%), in 667 species
Congruent 222,199 (80.4%)
Disparity >0 54,274 (19.6%)
Disparity >= 0.25 13,230 (4.8%)



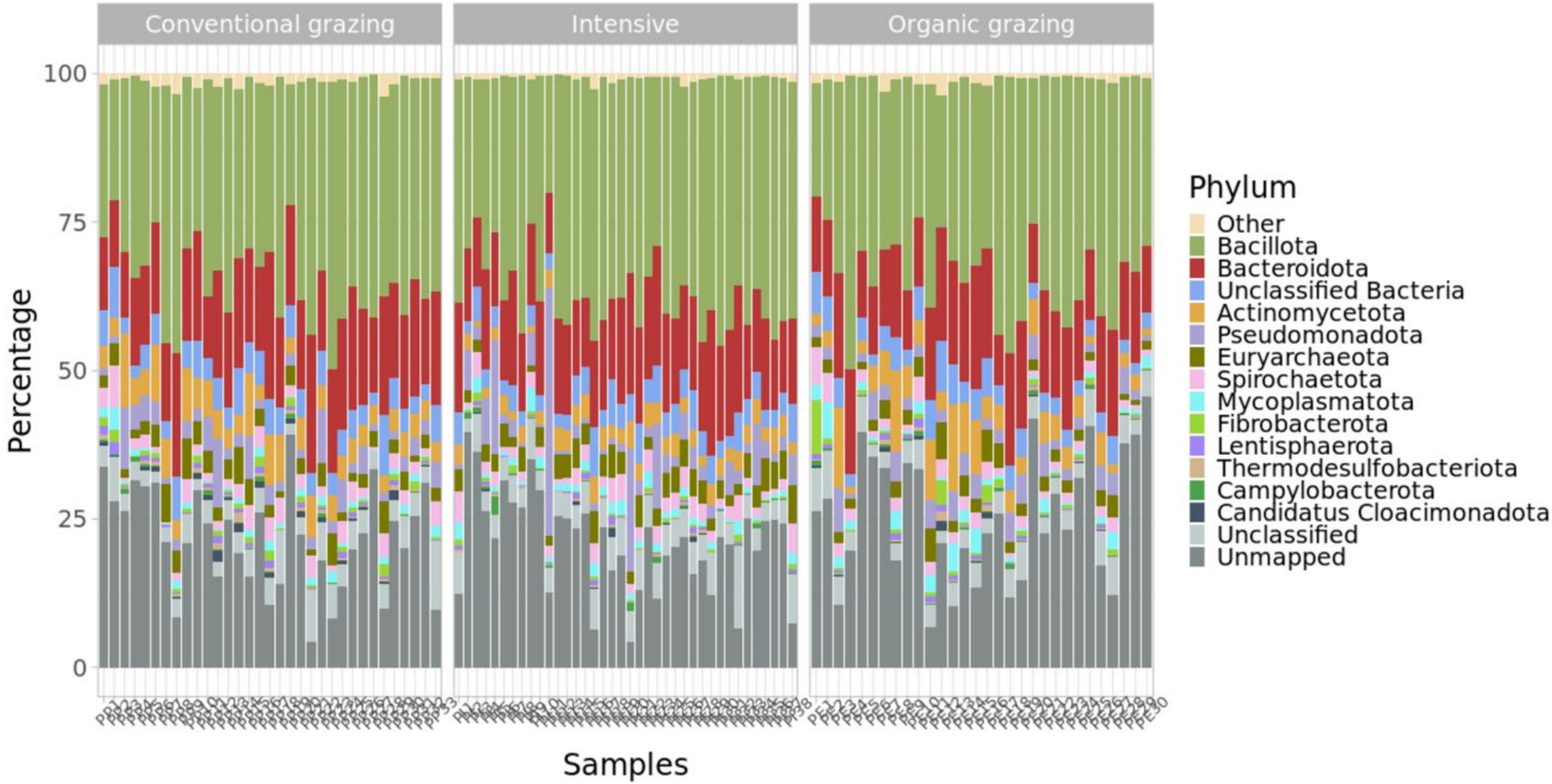
73% classified and mapped:

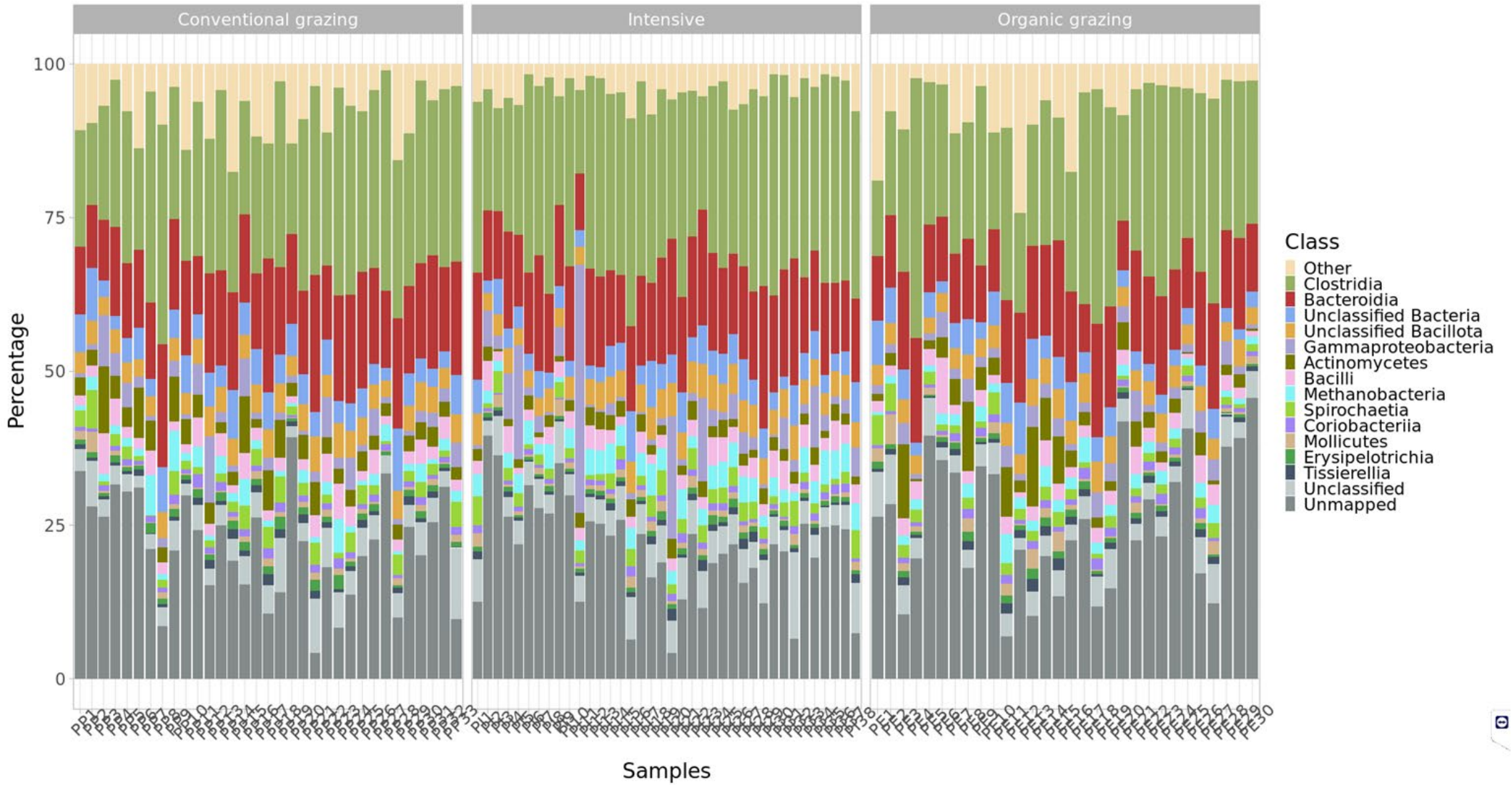
Archaea:4.61%

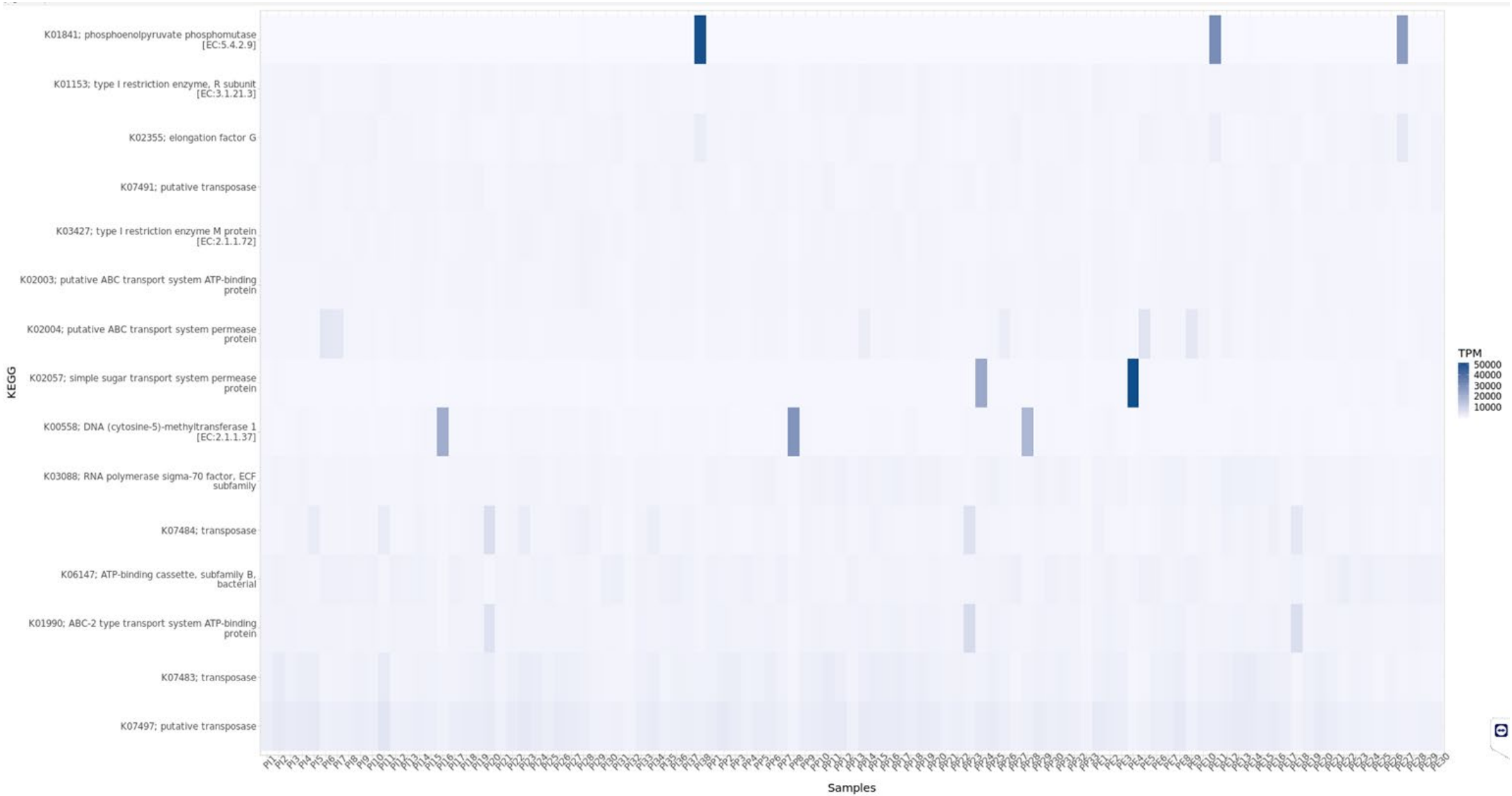
Bacteria: 95.22%

Eukaryota:0.07

Viruses:0.09


Conventional grazing
Intensive
Organic grazing
100
75
50
25
0
Percentage
Samples
Phylum
Other
Bacillota
Bacteroidota
Unclassified Bacteria
Actinomycetota
Pseudomonadota
Euryarchaeota
Spirochaetota
Mycoplasmatota
Fibrobacterota
Lentisphaerota
Thermodesulfobacteriota
Campylobacterota
Candidatus Cloacimonadota
Unclassified
Unmapped


Conventional grazing
Intensive
Organic grazing
100
75
50
25
0
Percentage
Samples
Class
Other
Clostridia
Bacteroidia
Unclassified Bacteria
Unclassified Bacillota
Gammaproteobacteria
Actinomycetes
Bacilli
Methanobacteria
Spirochaetia
Coriobacteriia
Mollicutes
Erysipelotrichia
Tissierellia
Unclassified
Unmapped


K01841; phosphoenolpyruvate phosphomutase [EC:5.4.2.9]
K01153; type I restriction enzyme, R subunit [EC:3.1.21.3]
K02355; elongation factor G
K07491; putative transposase
K03427; type I restriction enzyme M protein [EC:2.1.1.72]
K02003; putative ABC transport system ATP-binding protein
K02004; putative ABC transport system permease protein
K02057; simple sugar transport system permease protein
K00558; DNA (cytosine-5)-methyltransferase 1 [EC:2.1.1.37]
K03088; RNA polymerase sigma-70 factor, ECF subfamily
K07484; transposase
K06147; ATP-binding cassette, subfamily B, bacterial
K01990; ABC-2 type transport system ATP-binding protein
K07483; transposase
K07497; putative transposase
KEGG
TPM
50000
40000
30000
20000
10000
Samples

Conclusions

- Physicochemical analyses showed that there are significant differences for the slurry between the three differents livestock production systems regarding EC, TDS, DM, ammoniacal-N and K;

- The preliminary results of coassembly mode of slurry samples showed that 73% of microorganisms were classified and mapped and mainly formed by bacteria (95%) and the most abundant phylum are Bacillota, Bacteroidota and unclassified Bacteria.

- The sequential mode will be used in the further analysis to compare results with coassembly mode.

Acknowledgments

- Government of Cantabria; Regional Ministry for Rural Development, Livestock, Fisheries and Food;
- The Agricultural Research and Training Center of Cantabria;
- State Investigation Agency; FPI2019 aid for pre-doctoral researchers.

Thank you for your attention