Contribution of rumen ciliates to ruminant digestive system:

Usefulness of specific genes sequences

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Energy distribution among barley parts

Part Plant:

Head : 40-60 %

- Starch 60-65 %

Stems: 20-40 %

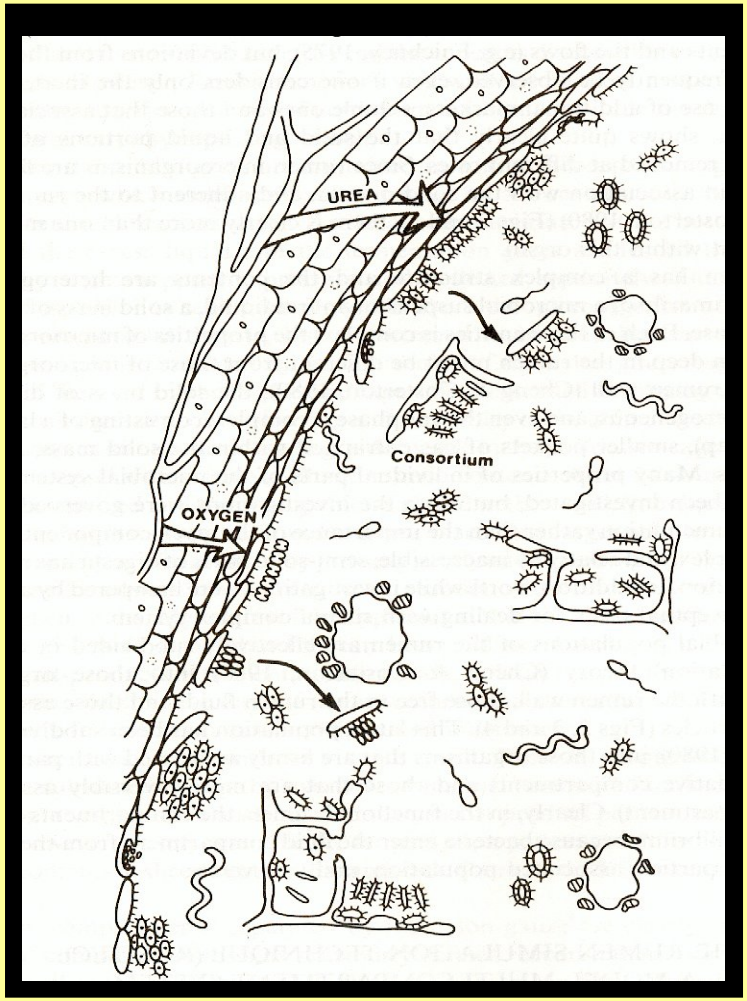
Leaves: 20-35 %

Carbohydrates

Structural Carbohydrates: 40-50 %

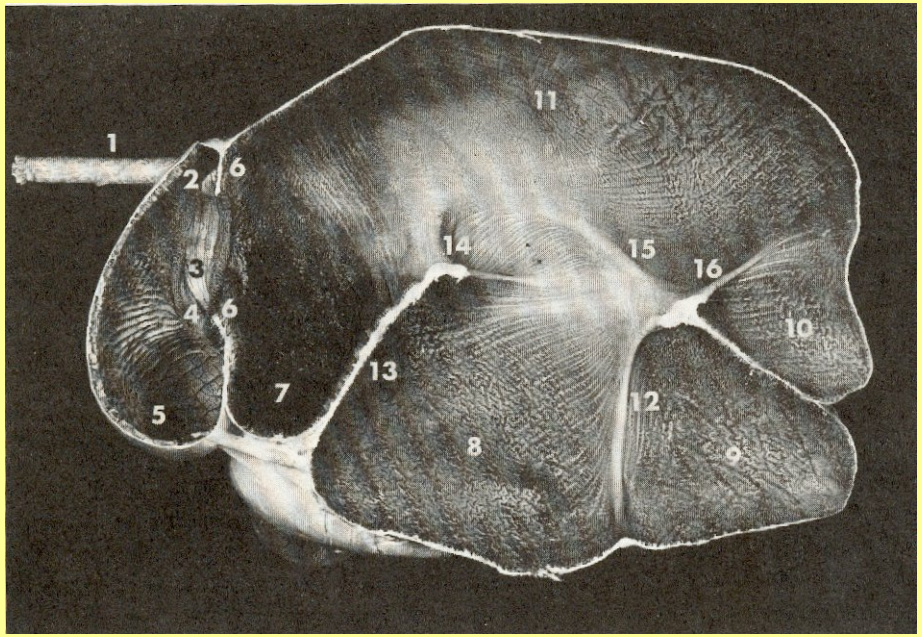
Non-Structural Carbohydrates: 50-60 %

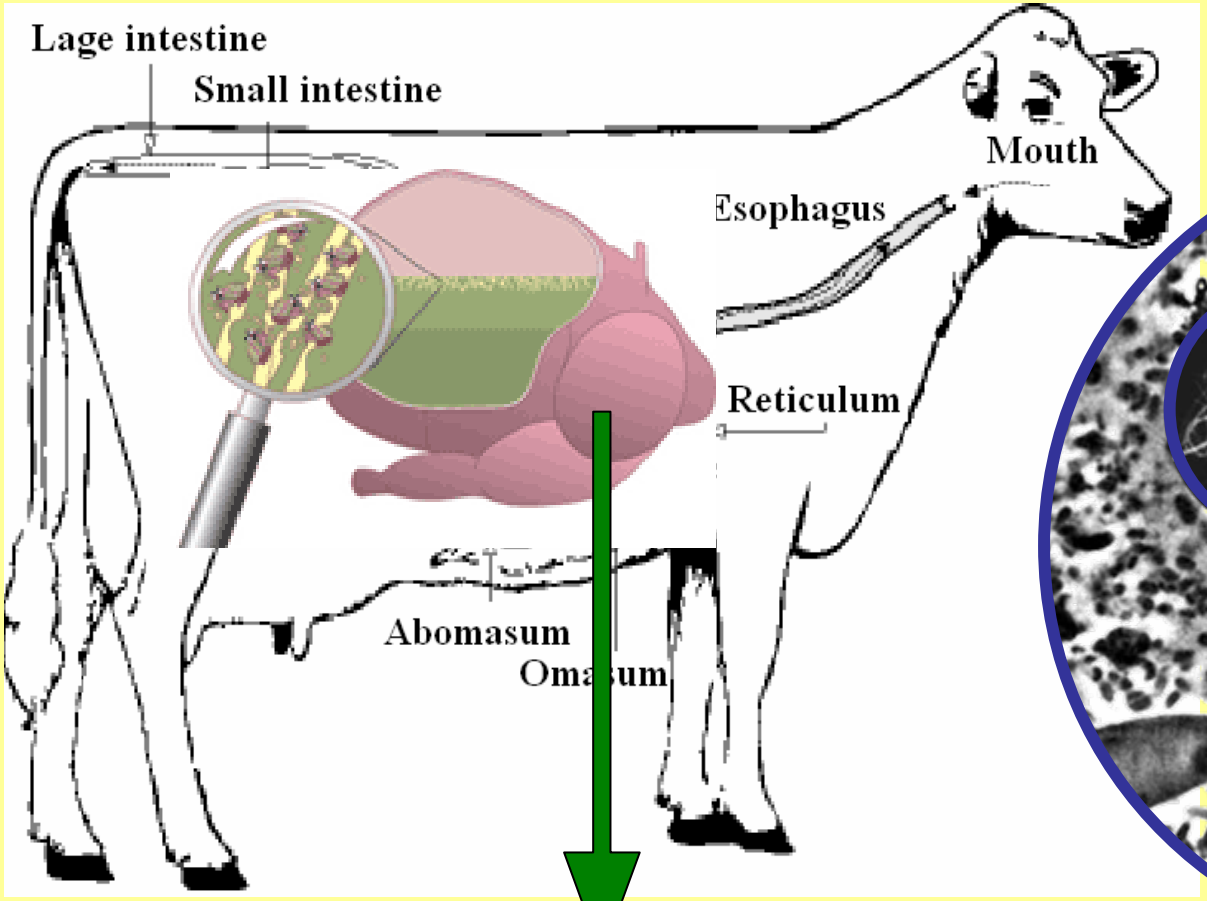
Herbivorous Symbiosis



ECOSYSTEM

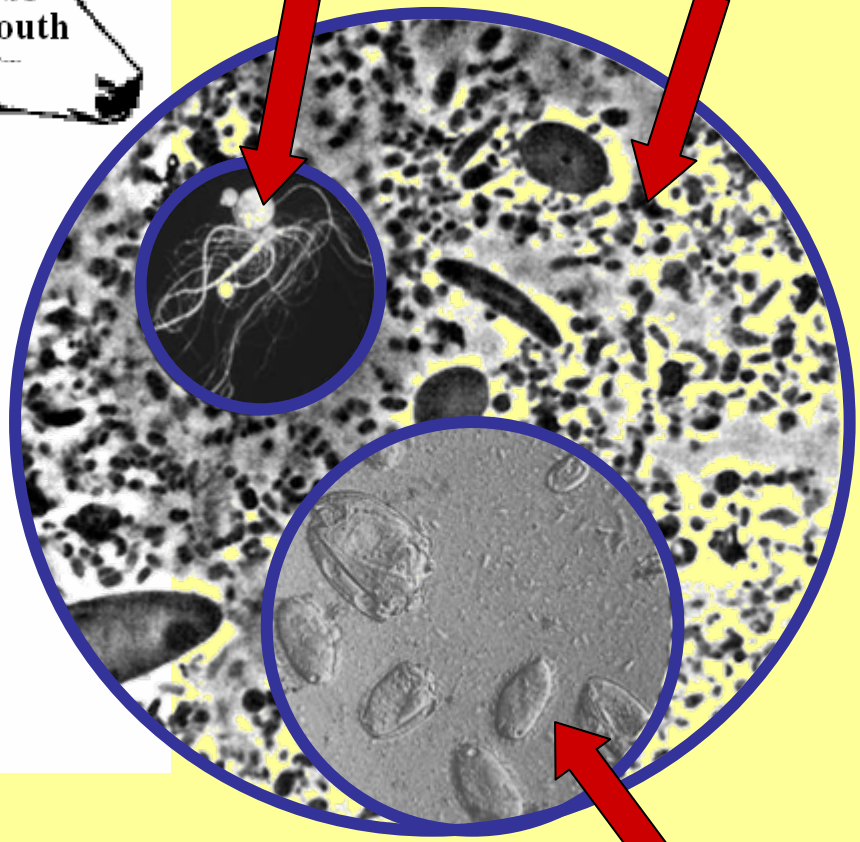
COMPARTMENT





Fungus
10^3-10^5/ml
5 genera
<8%DM

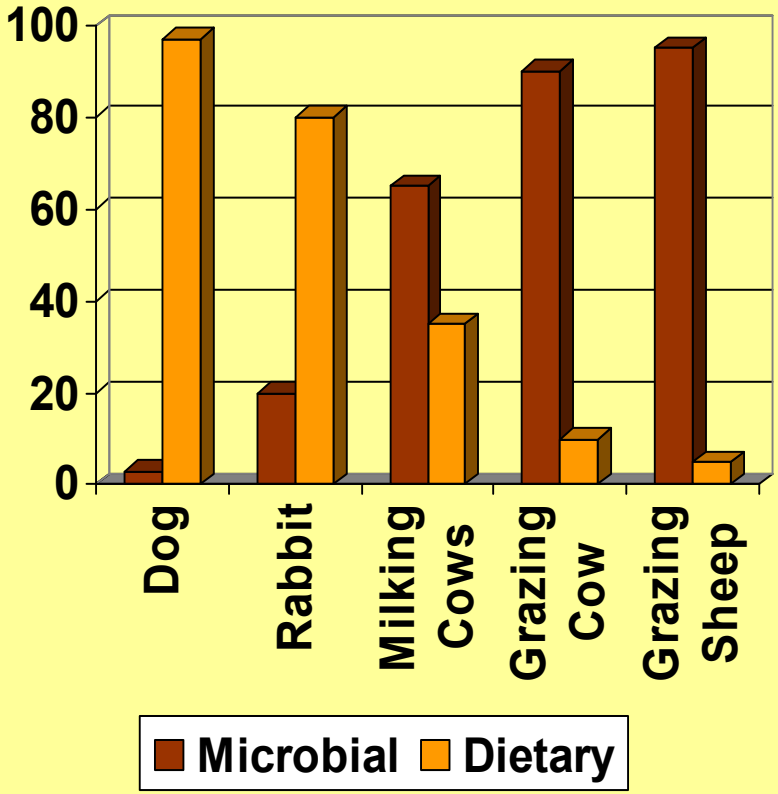
Bacteria
10^9-10^{11}/ml
200 species
<10 µm
60-90% DM



Protozoa
10^4-10^6/ml
100 species
10-200 µm
10-50%DM

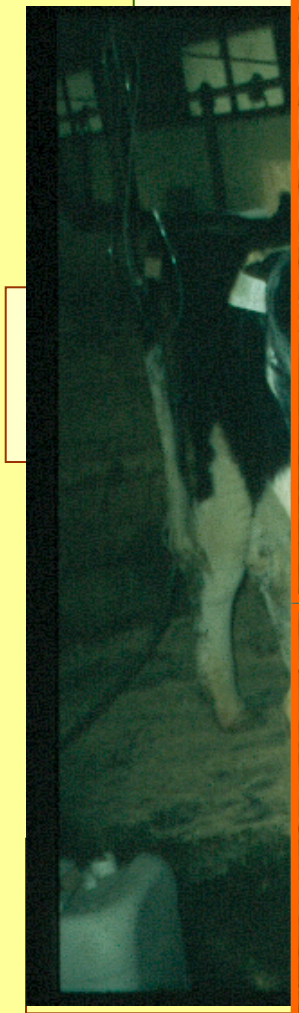
VFA
MICROBIAL PROTEIN

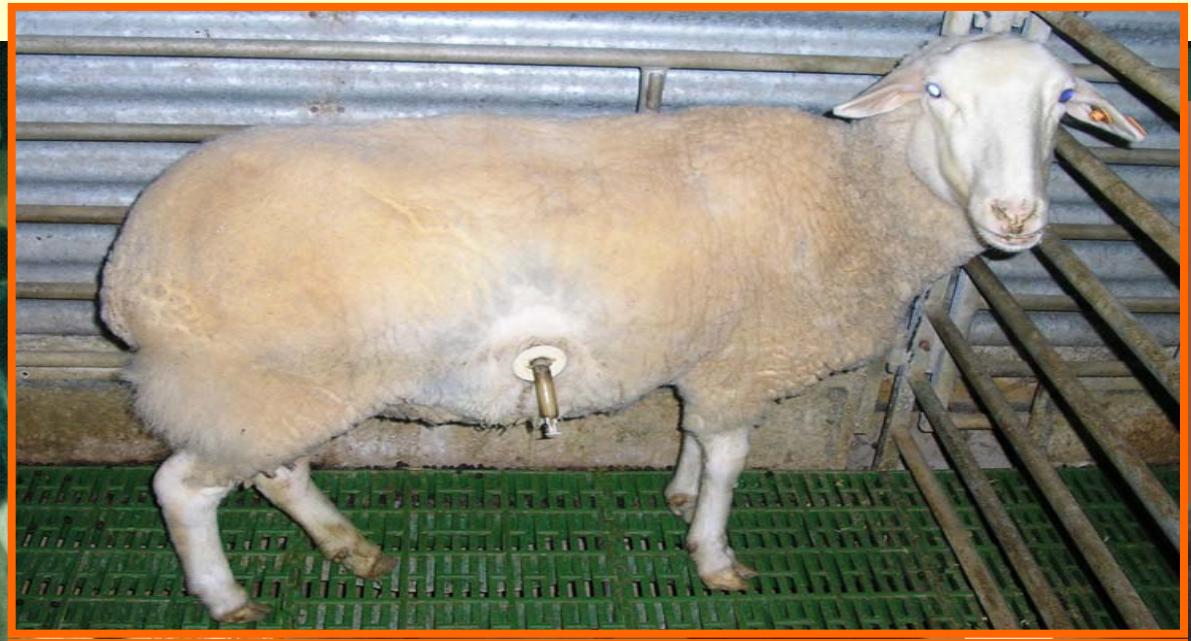
Microbial Contribution to AA Absorption

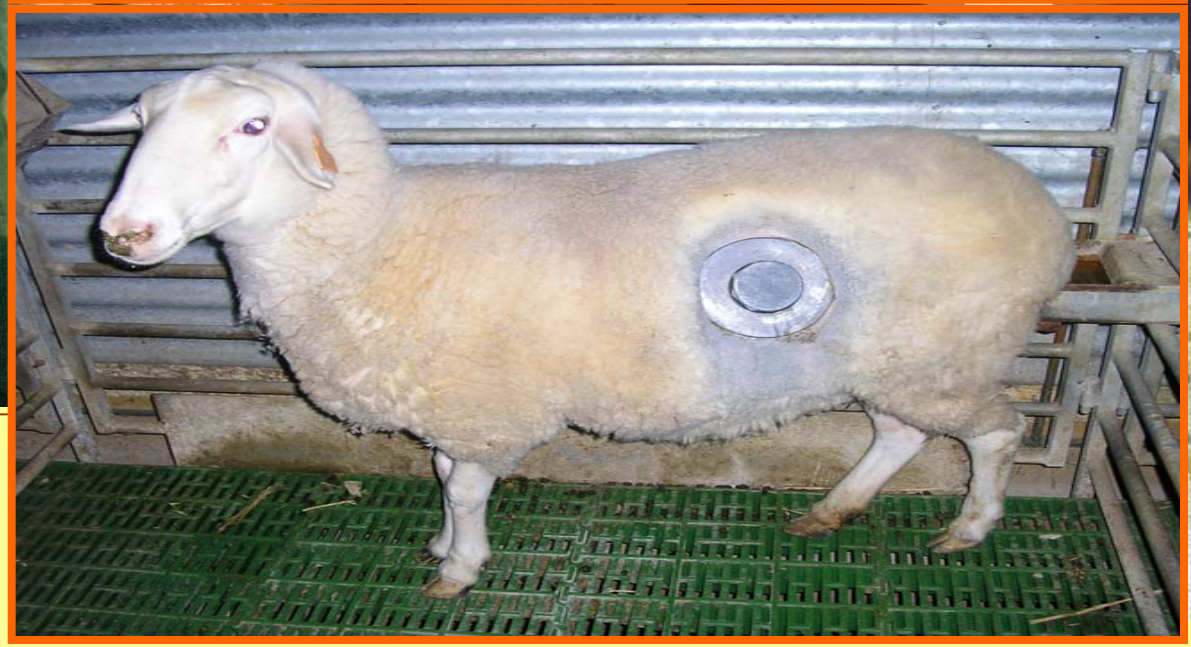


- Dog: 0 %
- Man: 5-10 %
- Rabbits: 15 -50 %
- Milking Cows : 50-80%
- Grassing Cows: 75-100%
- Grassing Sheep
  - Low quality grasses 100 %
  - Medium quality 80 %
  - High quality 65-75 %

Yield of Microbial Protein into the Rumen:







Yield of Microbial Protein into the Rumen: Conventional Methodologies

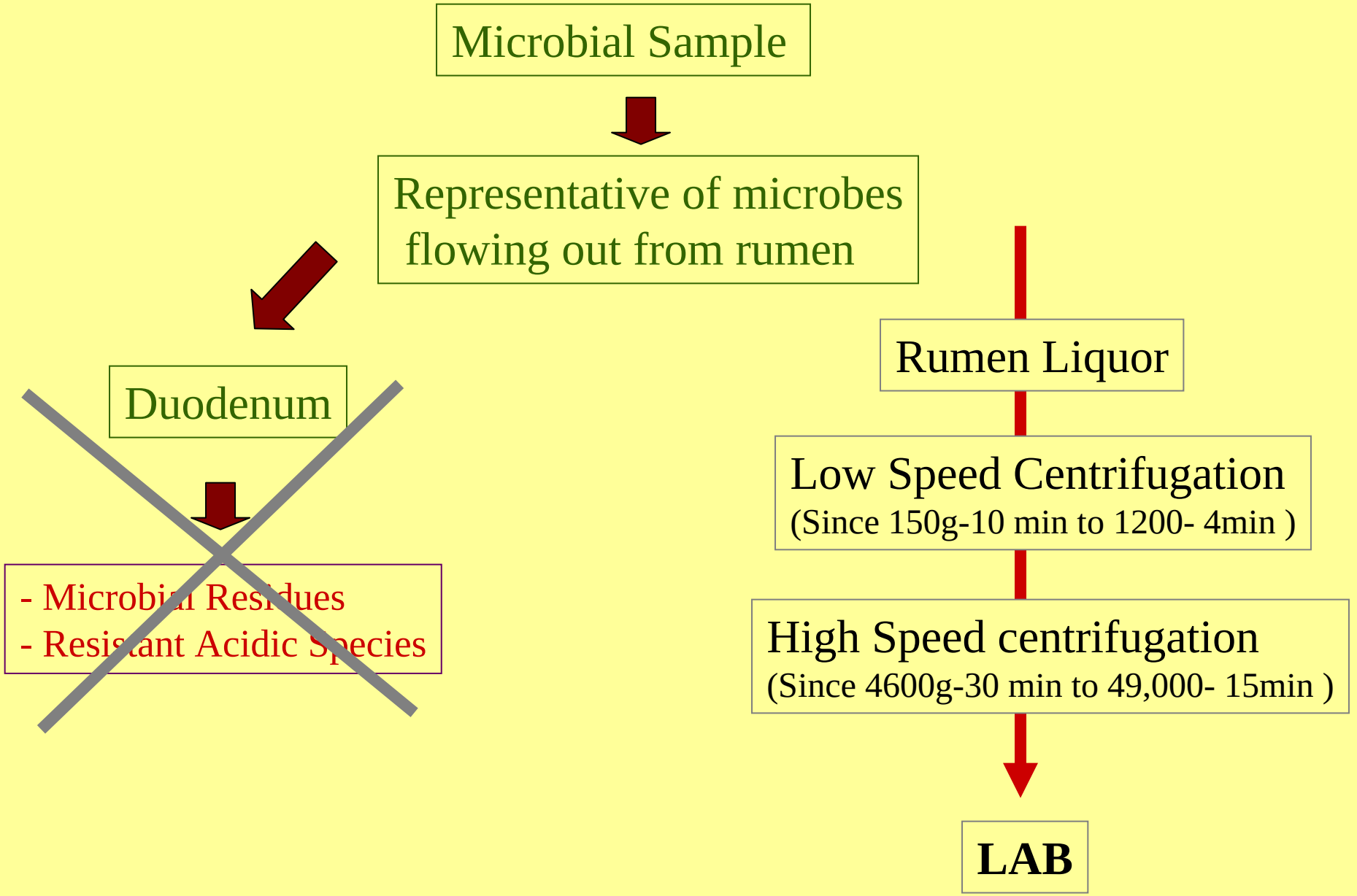
How much protein reach the duodenum?

Duodenal Canula:
- ***Re-entrant**
- ***T-Piece**
- ***Sacrifice**

How much is from microbial origin?

Microbial Marker
- *Natural
- *Isotopic

Representetive Microbial Sample
Rumen Canulation


Microbial Sample
Representative of microbes flowing out from rumen
Duodenum
- Microbial Residues
- Resistant Acidic Species
Rumen Liquor
Low Speed Centrifugation
(Since 150g-10 min to 1200- 4min)
High Speed centrifugation
(Since 4600g-30 min to 49,000- 15min)
LAB

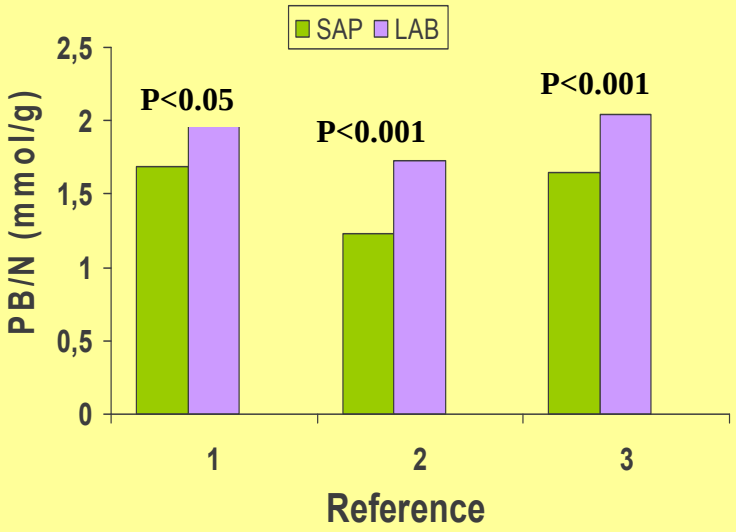
Liquid Associated Bacteria (LAB):

Are they representative of chymus population?

- Protozoa/fungui?

Lost Through
Low Speed Centrifugation

Solid Associated Bacteria?



1) Pérez, Balcells, Guada, Castrillo,1997. Anim. Sci., 65, 225-236

2) Martín-Orúe, Balcells, Zakraoui,Castrillo,1998, Anim.Feed Sci. Technol., 78, 269-282

3) Vicente, Guada, Balcells, Castrillo, 1999,Proc.BSAP, p.215. Abstract

Contribution to duodenal digesta of Non-Liquid Associated Micro-organisms

Are they relevant?

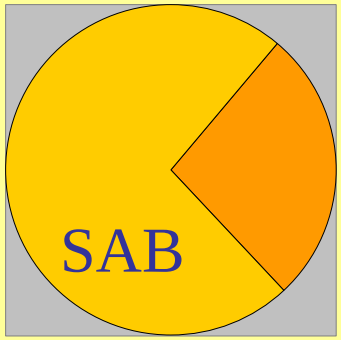
Protozoa
Fungi
SAB

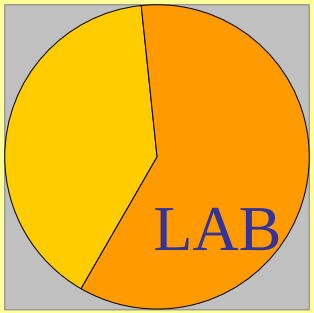
Significant and related to diet

No idea

Roughage diet:
NaOH-Treated Straw

Mixed Diet:
60 % Barley/40% NaOH-T. Straw





Pérez & Balcells (1998) Aust. J. Agri. Res. 49, 907

Conventional Microbial Markers

Inconvenient:

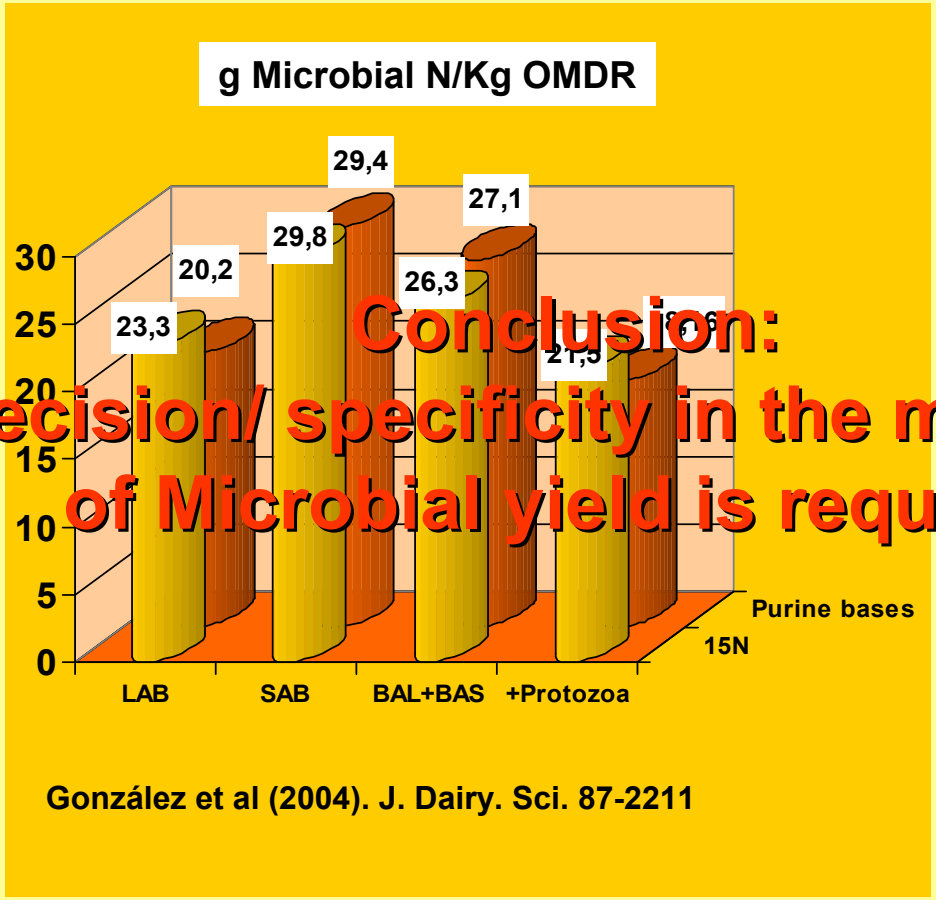
- Neither protozoa nor fungi markers does exist
- Low specificity of bacterial markers:
  - Marker/N ratio changes in function of microbial extracts, diet, food level, etc.
  - The impossibility to isolate a representative sample of microbes flowing out from rumen

Consequence:

- High variability on microbial protein synthesis estimations

Then, the question would be?
How much microbes are produced in an specific situation?

The answer: How much do you need?

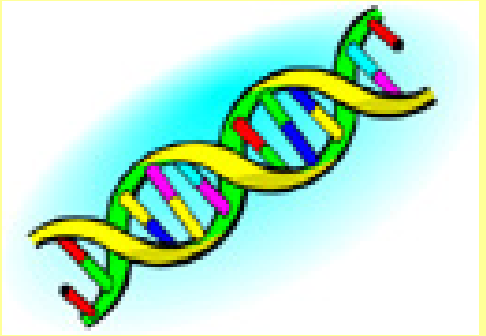


González et al (2004). J. Dairy. Sci. 87-2211

Conclusion:

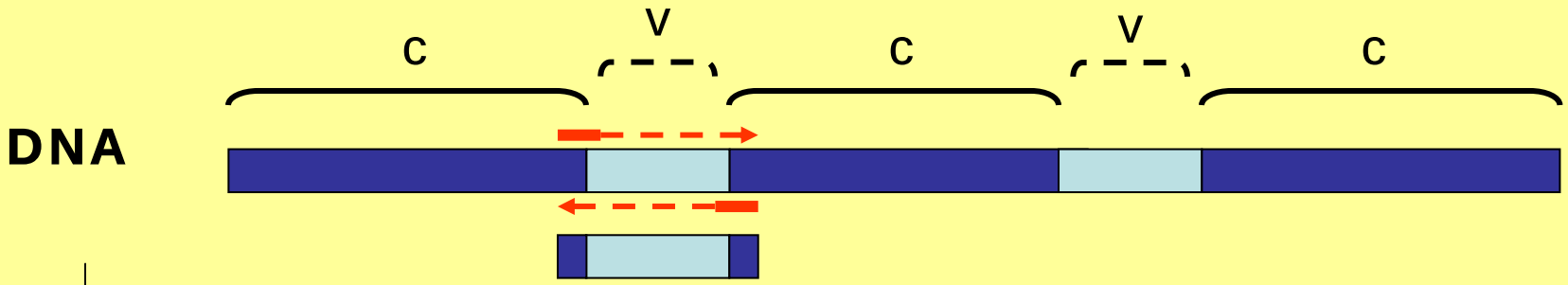
More precision/ specificity in the measurements of Microbial yield is required.

Genetic Markers

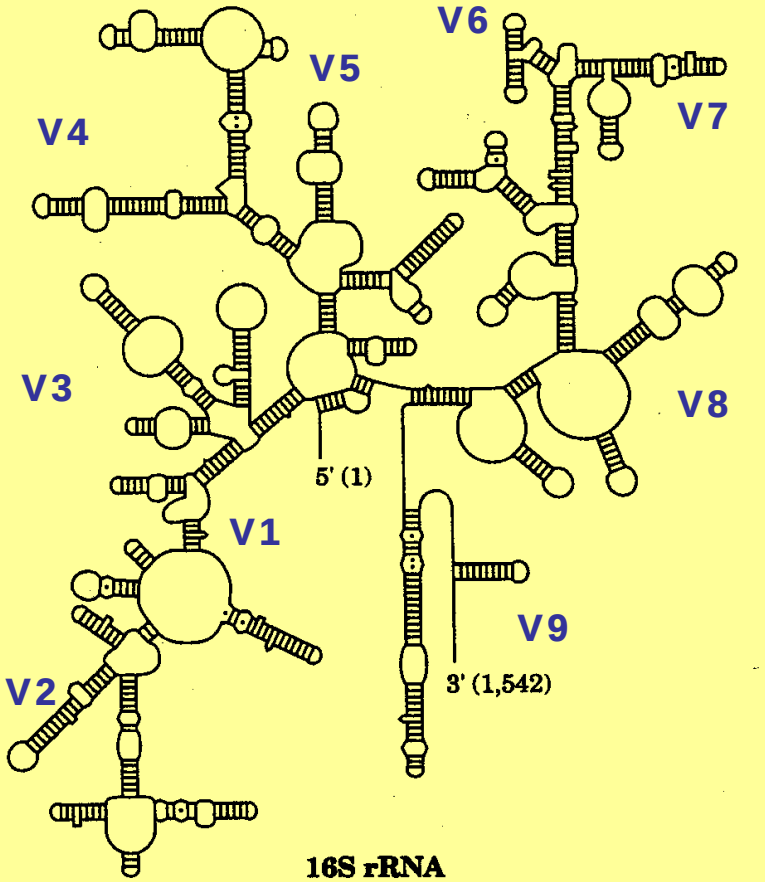


- ¿What are they?
  - ✓ Specific sequences of microbial DNA
- Possible advantages
  - ✓ High specificity
    - ✓ Bacterial marker (16S rDNA)
    - ✓ Protozoal marker (18S rDNA)
    - ✓ Specie-specific marker
  - ✓ Internal markers
  - ✓ Let study the rumen microbial ecosystem
  - ✓ They can represent microbes flowing out from rumen

Ribosome



RNAr



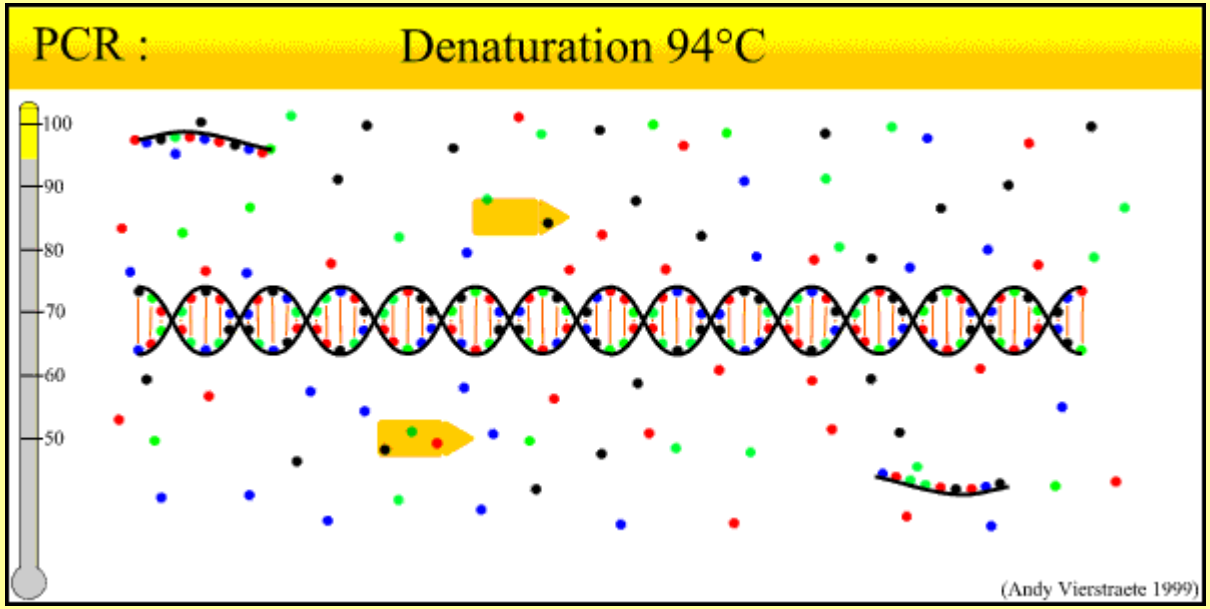
Requirements of the new approach

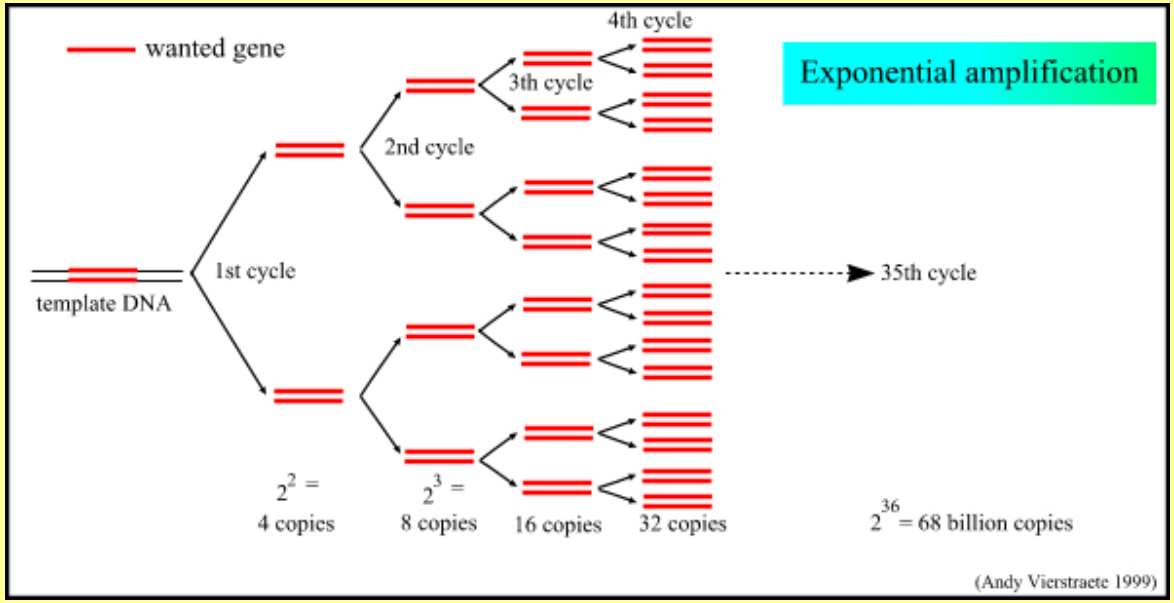
- Specific DNA sequences for rumen microbes does exist?
- We have the ability to amplify and quantify DNA-seq, PCR system?
- DNA-seq persist though abomasums digestion.
  - In vitro?
  - In vivo?
- They behave like the conventional markers?

Microbial Quantification qPCR:
Available primers

Microbes	Reference
•Entodinium y Dasytricha spp	•Skillman et al 2006 Appl Environ Microbiol 72, 200-6
•Protozoa	•Sylvester et al 2005 J Dairy Sci 88, 2083-95.
•Total Bacterias	•Maeda et al 2003 FEMS Inm Med Microbiol 39, 81-86
•Megasphaera elsdenii YE 34 & Butyrivibrio fibrisolvens YE 44	•Klieve et al 2003Appl Microbiol 95, 621-30.
•Prevotella ruminicola, Prevotella albensis, Prevotella bryantii, Fibrobacter succinogenes, Selenomonas ruminantium-Mitsuokella multiacida, Streptococcus bovis, Ruminococcus flavefaciens, Ruminobacter amylophilus, Eubacterium ruminantium, Treponema bryantii, Succinivibrio dextrinosolvens, and Anaerovibrio lipolytica.	•Tajima et al 2001 Appl Environ Microbiol 67, 2766-74.
•Fungi	•McSweeny et al 2006 (en prensa)
•Metanogénic Archeas	•McSweeny et al 2006 (en revision) Appl Environ Microbiol

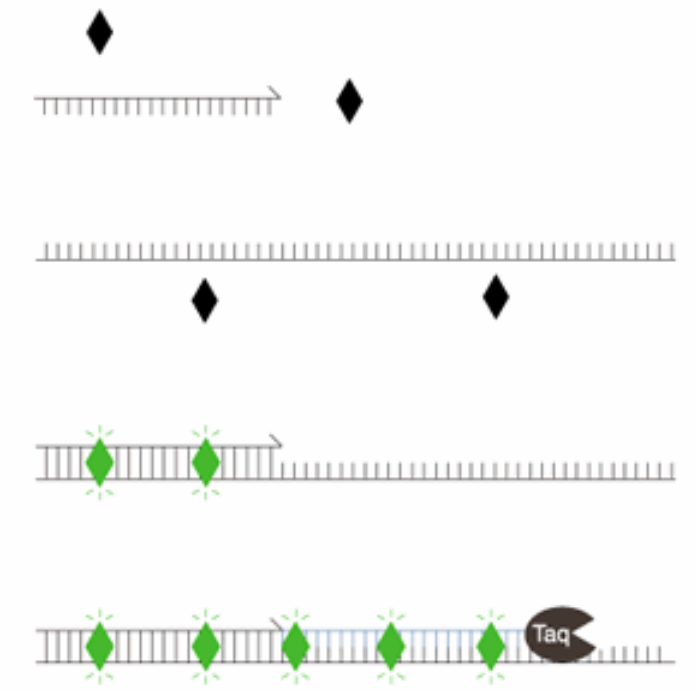
PCR

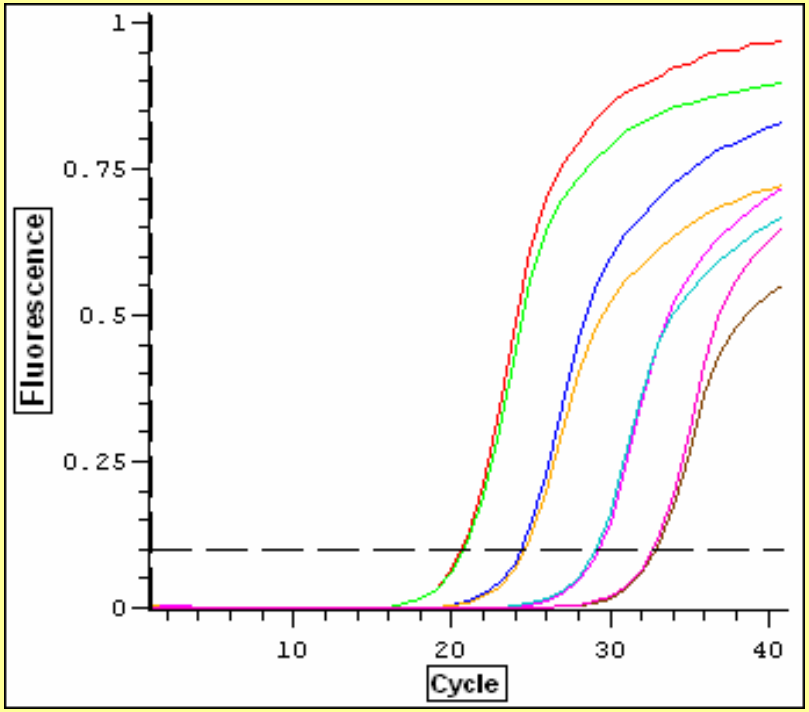

PCR : Denaturation 94°C
100
90
80
70
60
50
(Andy Vierstraete 1999)


wanted gene
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
Exponential amplification
35th cycle
2^2 = 4 copies
2^3 = 8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)

Microbial Quantification

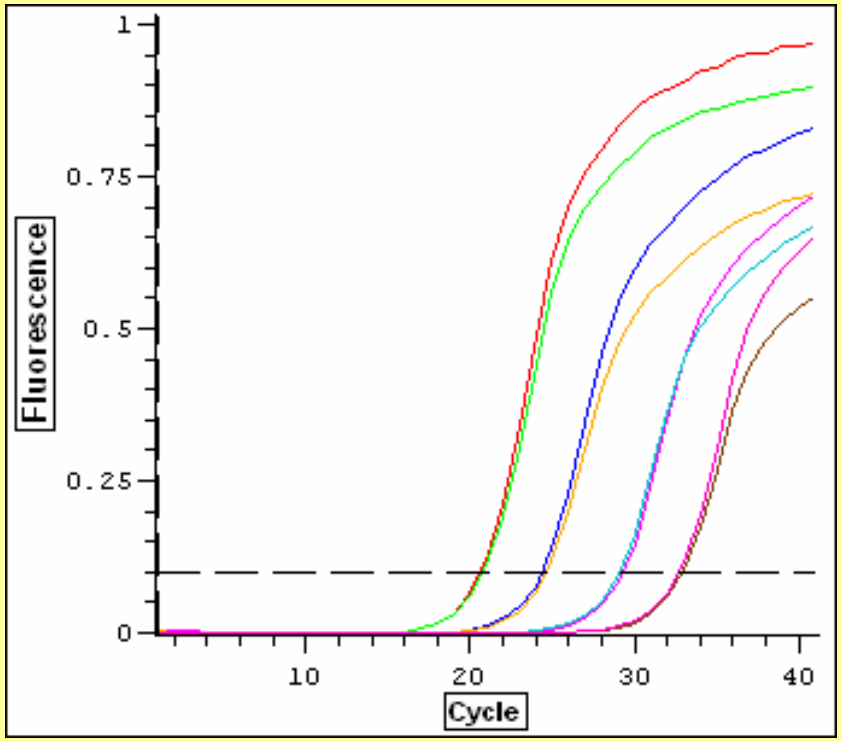
qPCR- Real Times

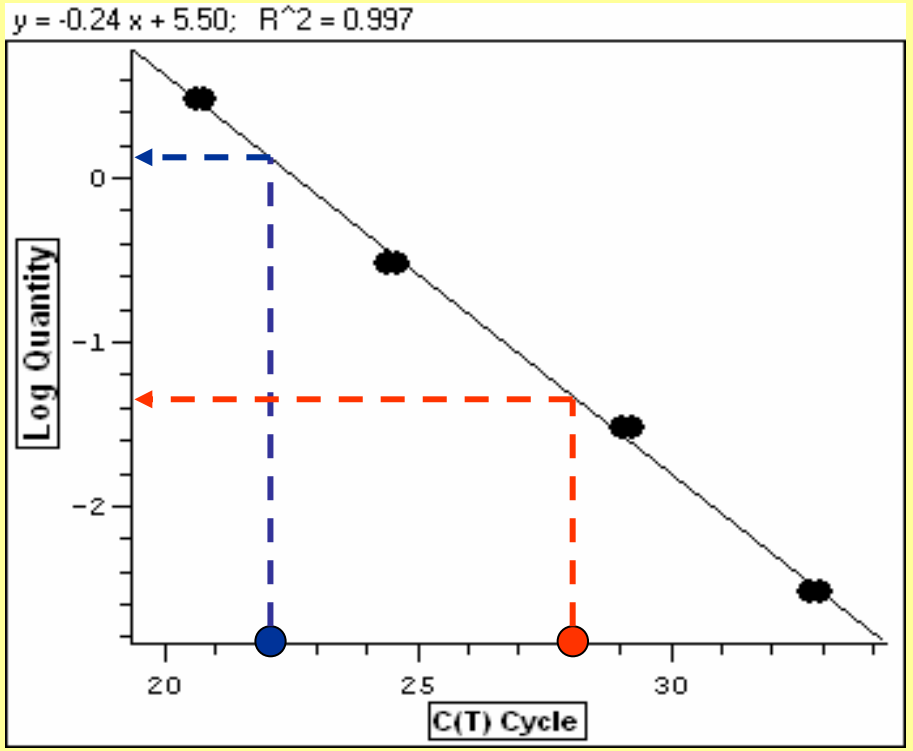




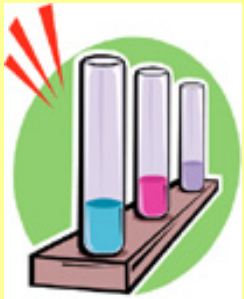
Microbial Quantification

PCR - Real Times





In vitro survival of specific DNA-Seq

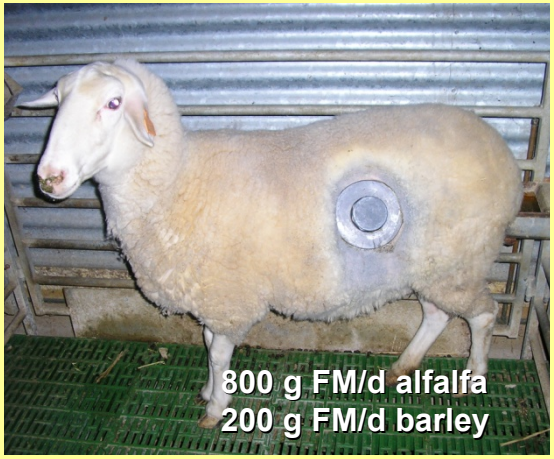


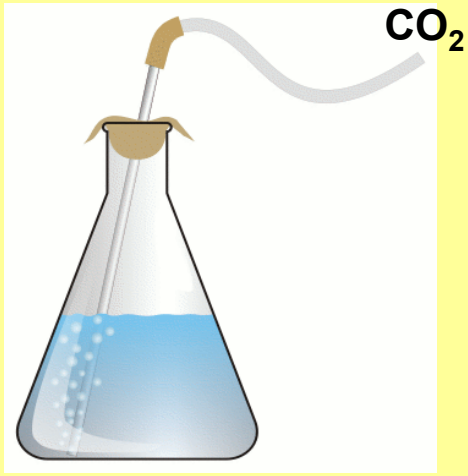
Objectives:

- To determine survival of specific DNA-sequences within abomasums conditions

- Study of DNA-sequences vulnerability from different microbes and microbial species.

Methodology





Liquid associated bacteria

Rumen Protozoa

Sedimentation
Differential centrifugation
Filtration
Washing

(Sylvester *et al.*, 2004)

Incubation

Incubation conditions

HCl

- 200mM (pH 1.2)
- 85mM (pH 2.3)
- 55mM (pH 4.2)

Fibre

- 0 g/l CMC
- 2 g/l CMC

Pig pepsin

- 0.6 g/l (1.700 units/l)
- 1.8 g/l (5.100 units/l)

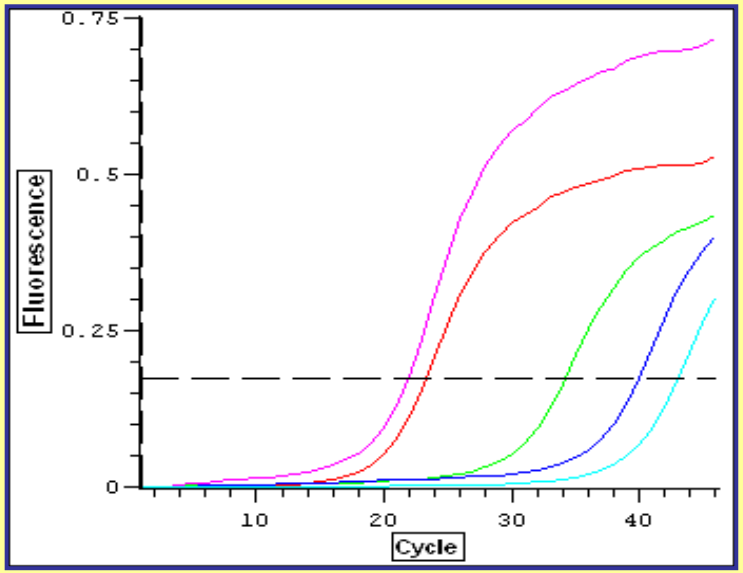
Incubation time (39ºC)

- Inoculums
- 20 min
- 40 min
- 60 min

DNA extraction and quantification

DNA Extraction

- Kit Qiagen®

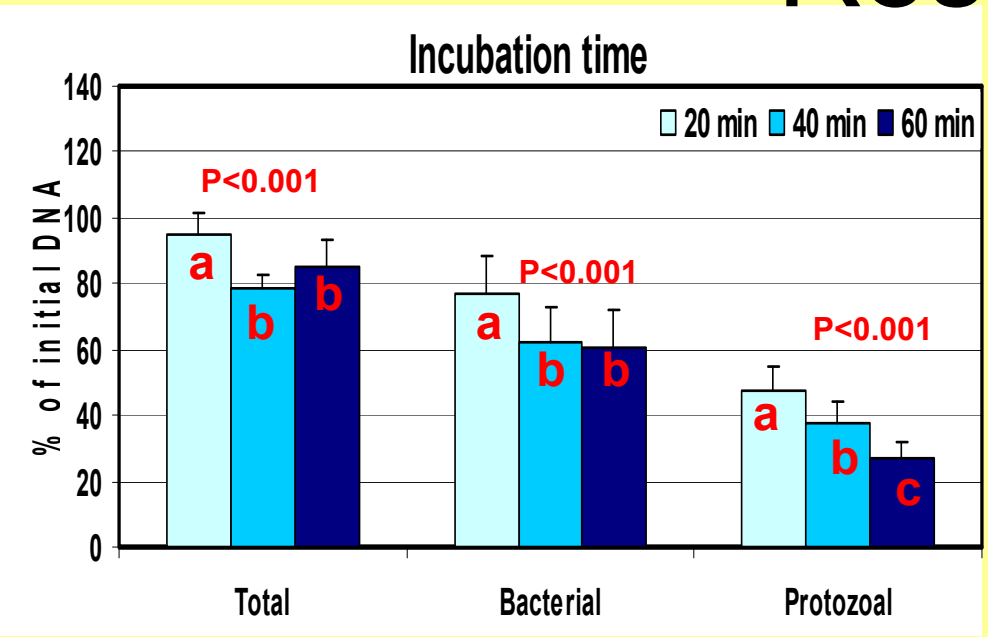


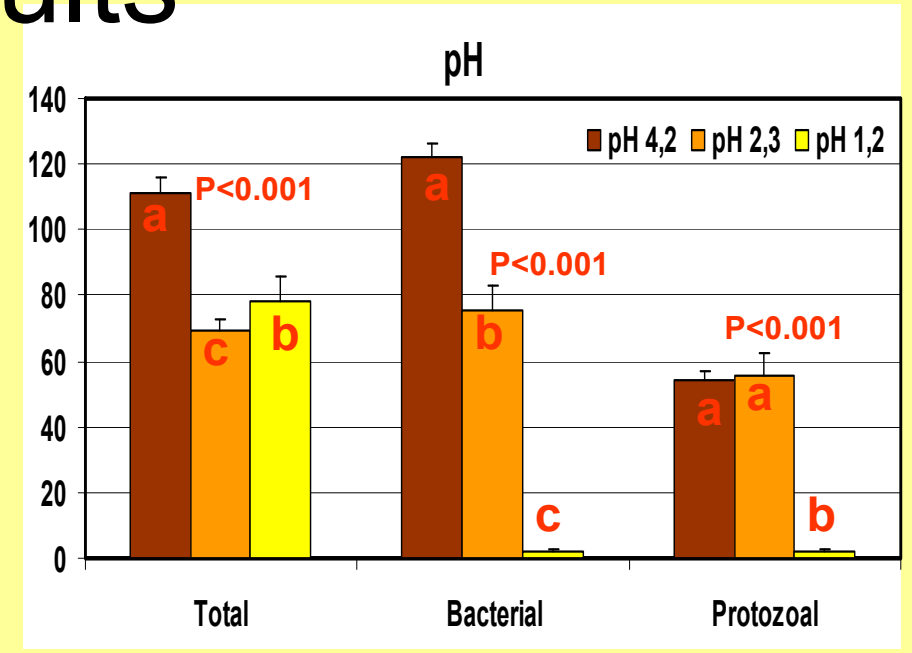
DNA quantification

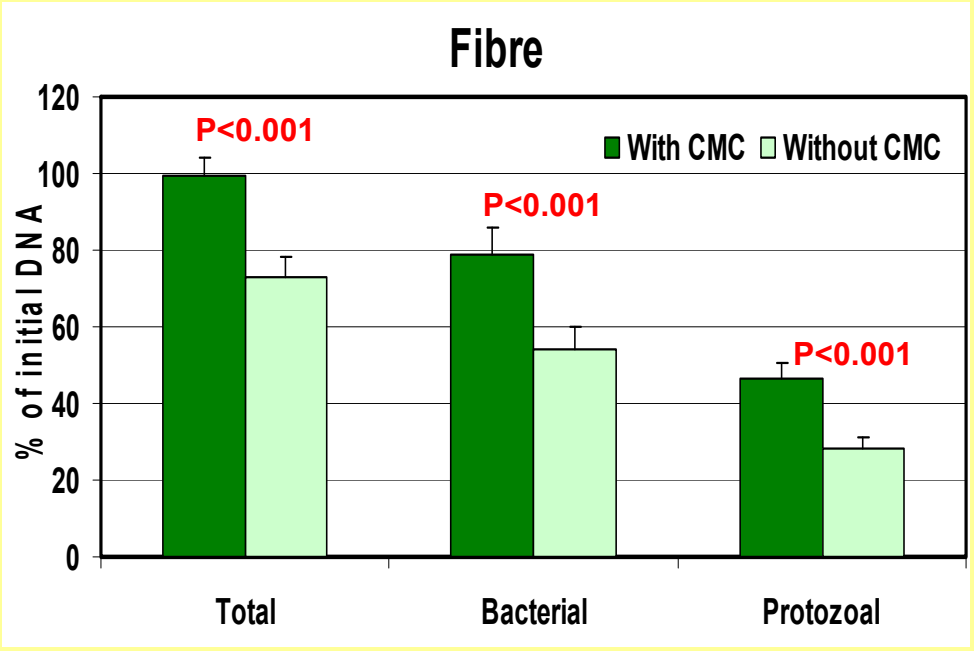
Total DNA → **Spectrophotometer**

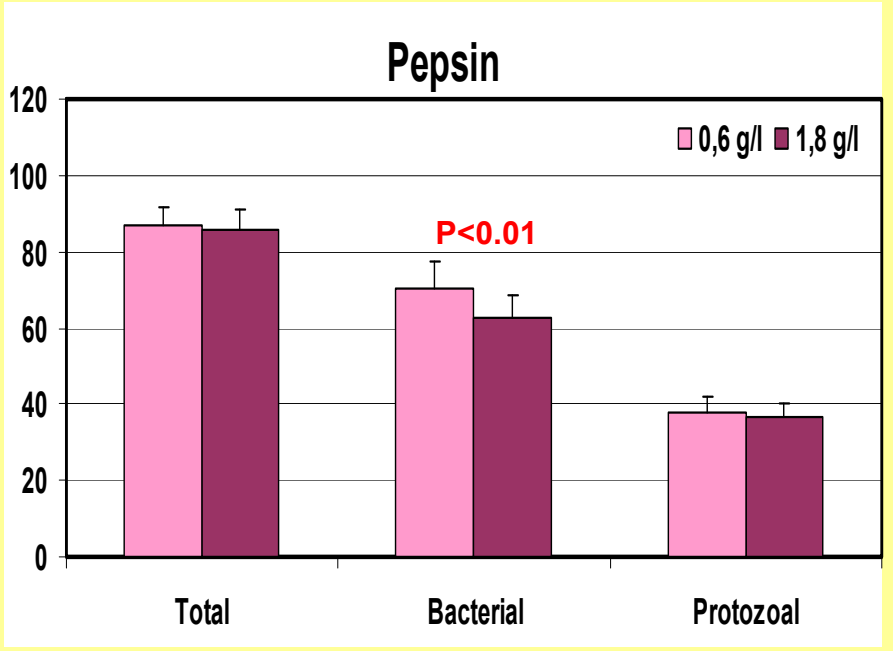
Microbial DNA → **Real time PCR** { **Specific primers** / **SYBR-Green**

Results

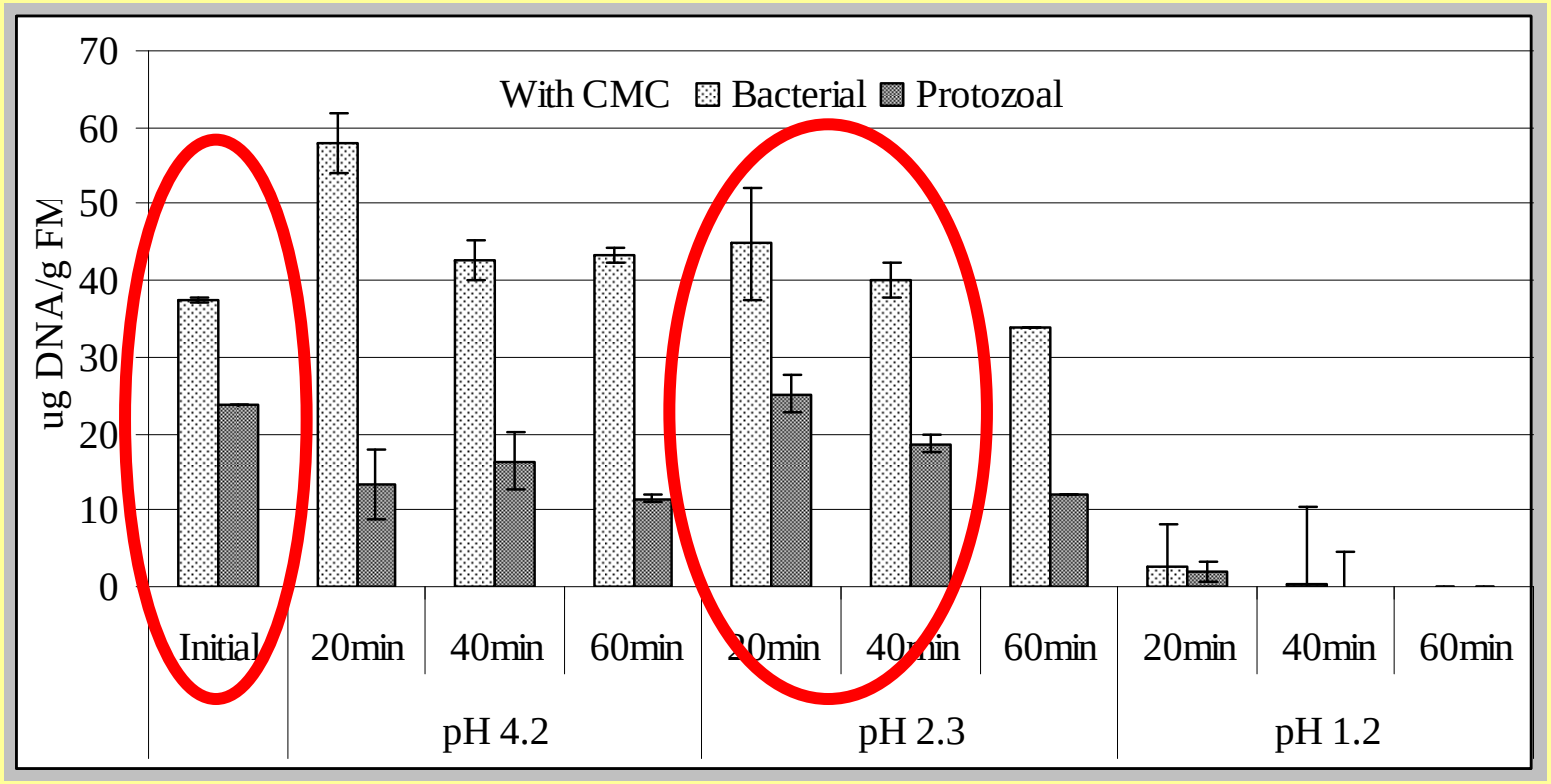

Incubation time
% of initial DNA
20 min
40 min
60 min
P<0.001
P<0.001
P<0.001
a
b
b
a
b
b
a
b
c
Total
Bacterial
Protozoal


pH
pH 4,2
pH 2,3
pH 1,2
P<0.001
P<0.001
P<0.001
a
c
b
a
b
c
a
a
b
Total
Bacterial
Protozoal

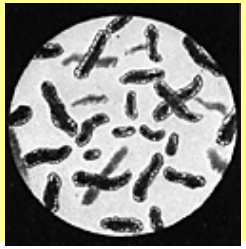

Fibre
% of initial DNA
With CMC
Without CMC
P<0.001
P<0.001
P<0.001
Total
Bacterial
Protozoal

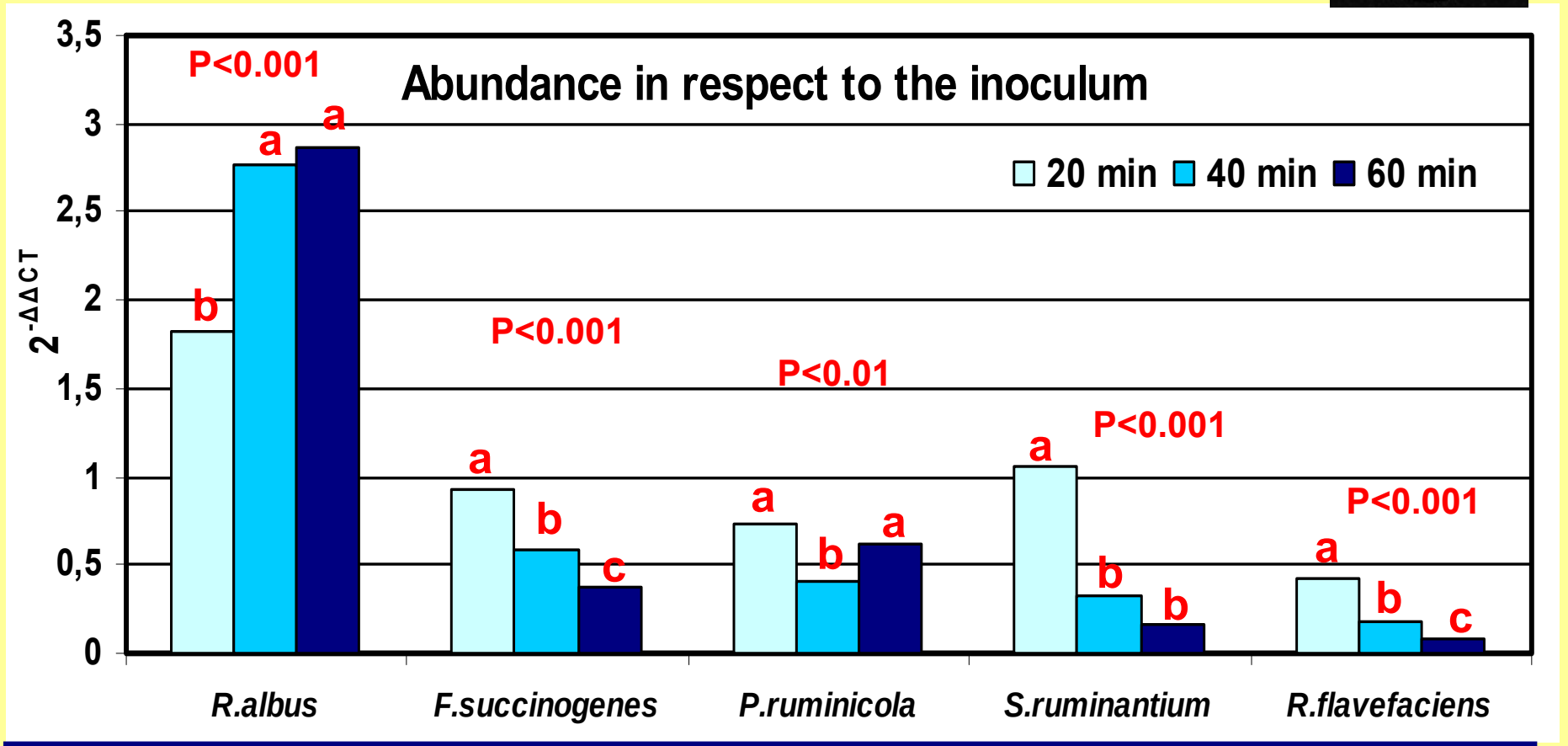

Pepsin
0,6 g/l
1,8 g/l
P<0.01
Total
Bacterial
Protozoal

Concentrations (µg/g FM) of bacterial and protozoal DNA-seq quantified by q-PCR incubated at various pH (4.2, 2.3, or 1.2) with fibre presence (carboxymethylcellulose,2 g/l) and incubated at 20, 40, or 60 min.



Efect of digestion at pH 2.3 on DNA from several bacterial species





Gram:	positive	negative	negative	negative	positive
Activ:	Cellulolitic	Cellulolitic	Amylolitic	Amylolitic	Cellulolitic
Amplicon:	175 pb	446 pb	485 pb	513 pb	835 pb

Implications

- Under simulated physiological abomasums conditions a high (≈ 100%) proportion of the bacterial gene sequences used as markers kept the molecular integrity

- Protozoa gene sequences were more susceptible to be digested and only around 75% of the protozoa DNA survive abomasums digestion

- Comparing the degradation of G(+) [R. albus and R. flavefasciens] against Gram(-)bacterial species [*F. succinogenes, P. ruminicola, S. Ruminantium* no differences were observed. However, abomasums persistence may be related to the amplicon size, small size showed lesser probability to be digested.

In vivo survival of specific DNA-Seq

Objectives

- To study survival of Specific DNA- Seq through lambs digestive tract.

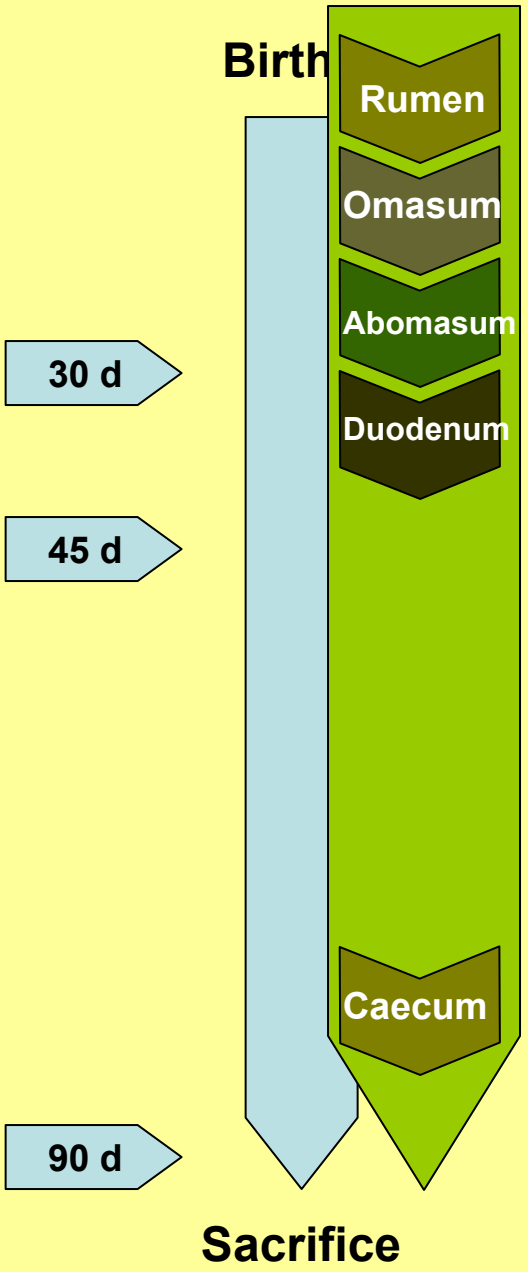
- To analyse *in vivo* DNA-seq as potential microbial marker.

The Specific DNA fractions can be detected through digestive tract of lambs?

When?

Where?

How much survive trough digestion?



Methodology

Milk diet (30d)- 8.5kg

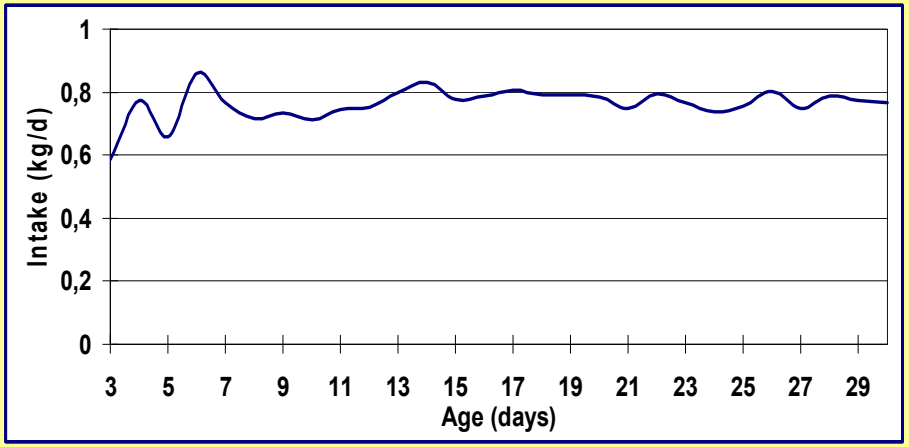
Milk
8,5kg

Weaning age (45d)-15kg

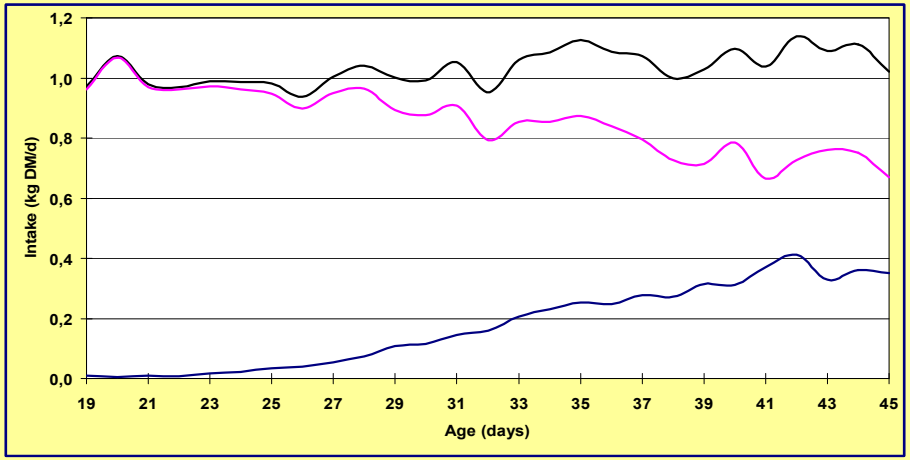
Milk
Concentrate
Straw

Fattening (90d)-24kg

Concentrate
Straw



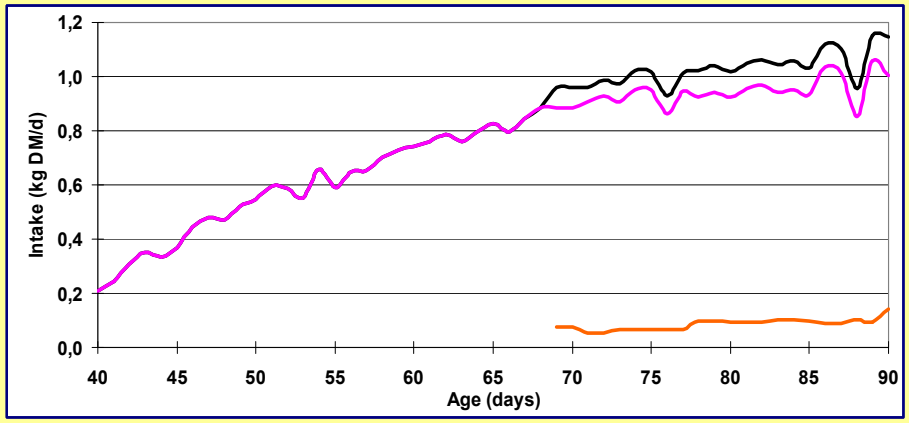
Milk
0,80 l/d



Total
1,07 kg/d

Milk
0,72 l/d

Concentrate
0,35 kg/d



Total
1,02 kg/d

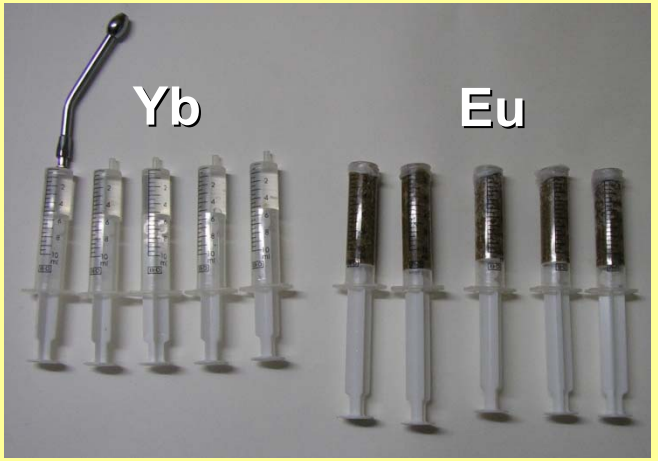
Concentrate
0,96 kg/d

Straw
0,10 kg/d

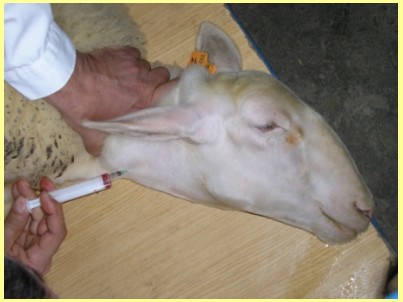
Methodology

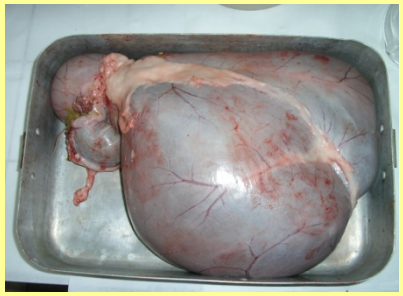
Flow markers

- 5 last days, 6 times/d
- Ytterbium chloride (2 mg/kg PV·d)
- Europium acetate (14 mg/kg PV·d)

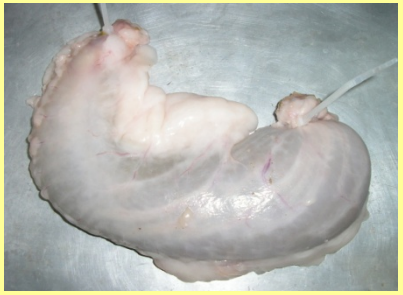


Slaughter





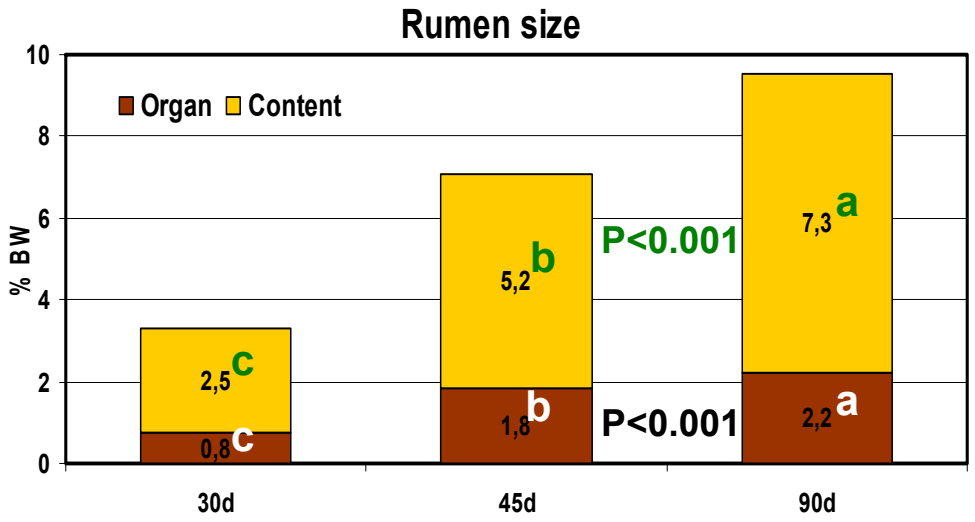
Rumen

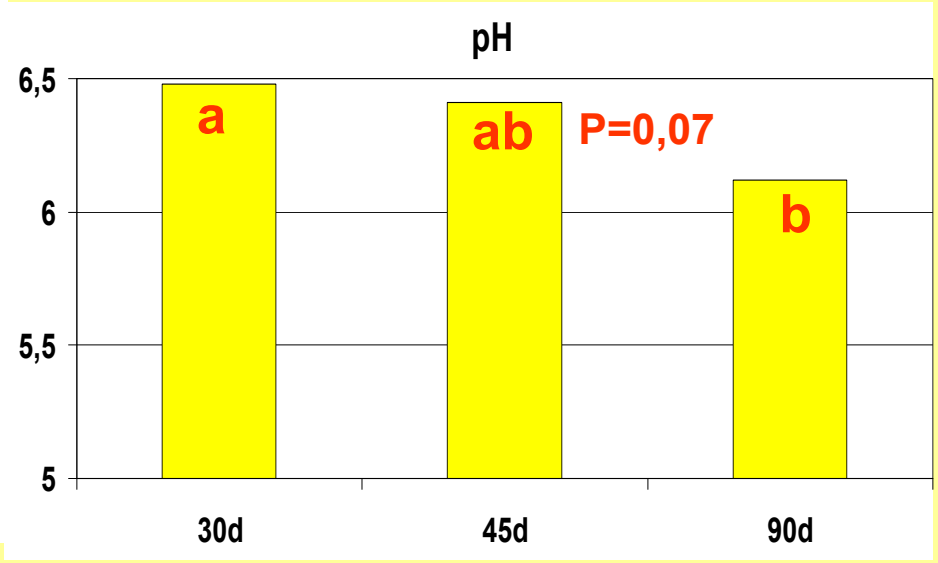


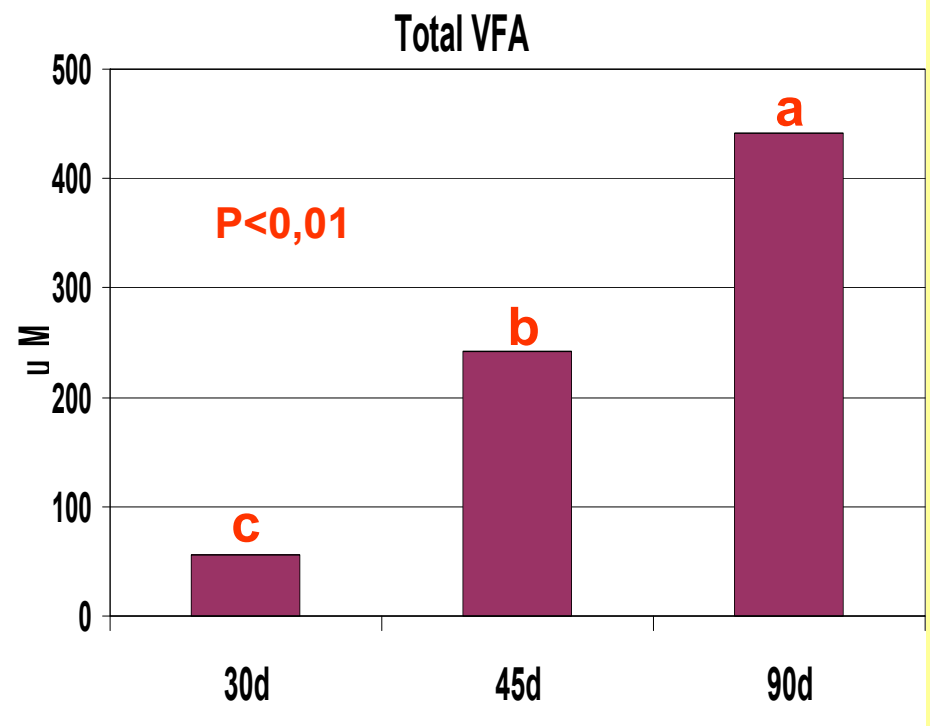
Abomasum

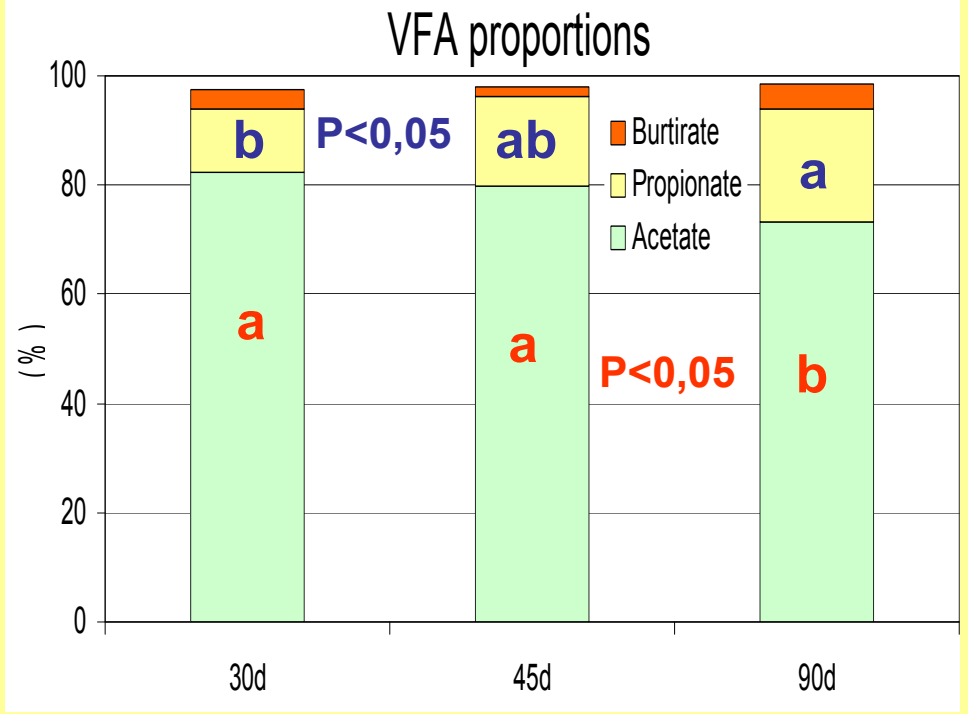
Duodenum

Rumen development

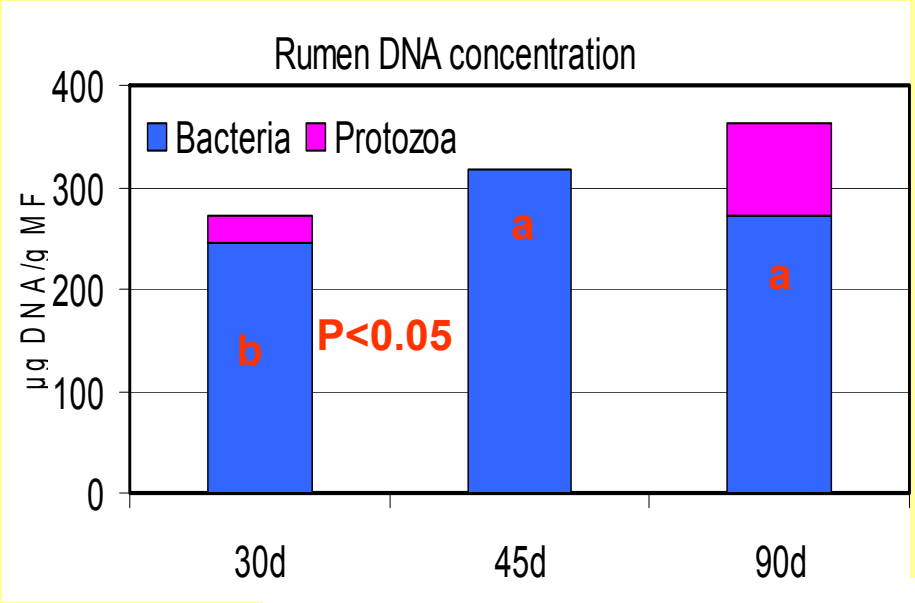


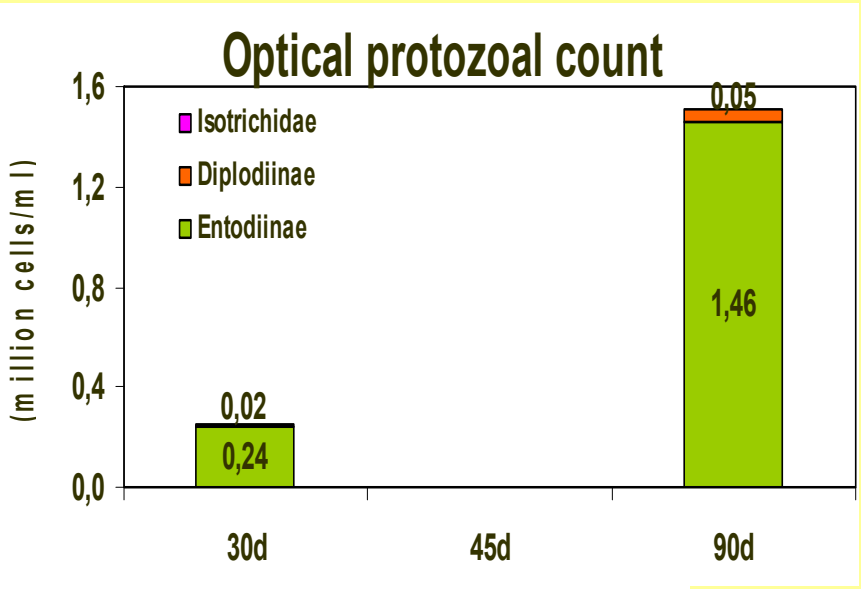


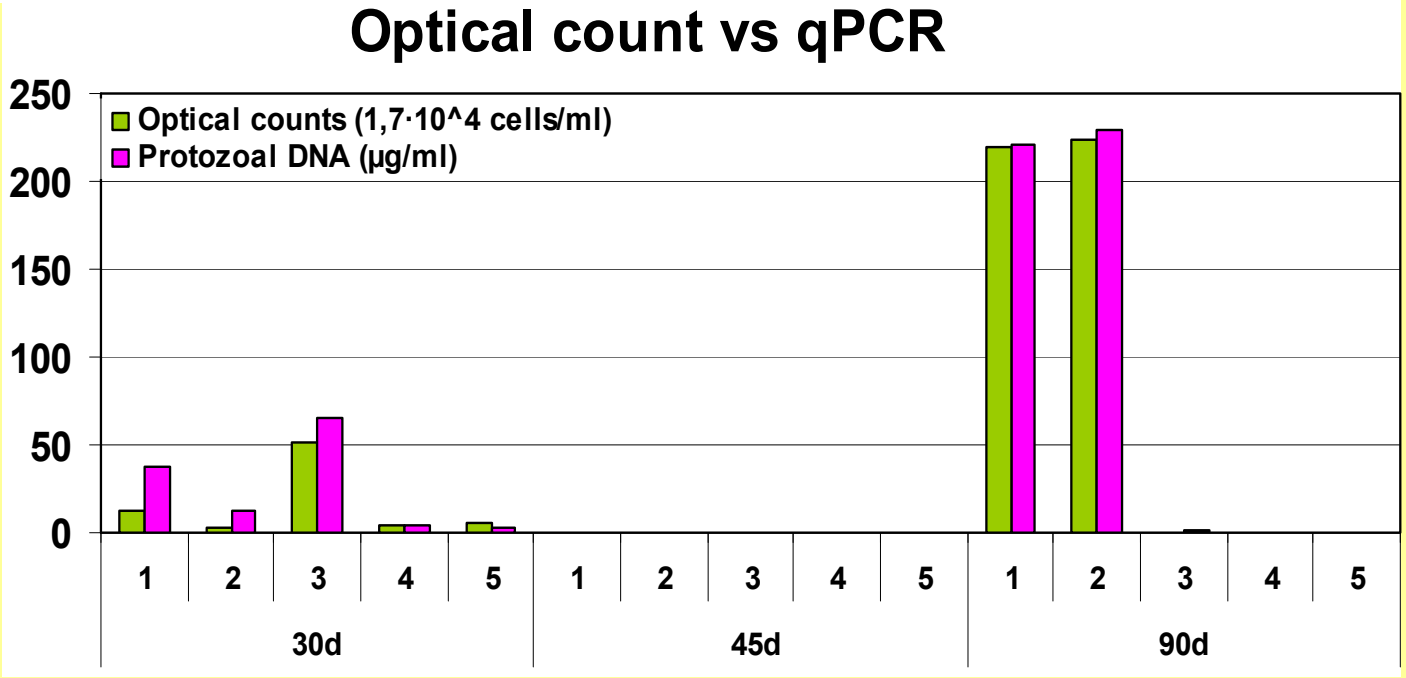




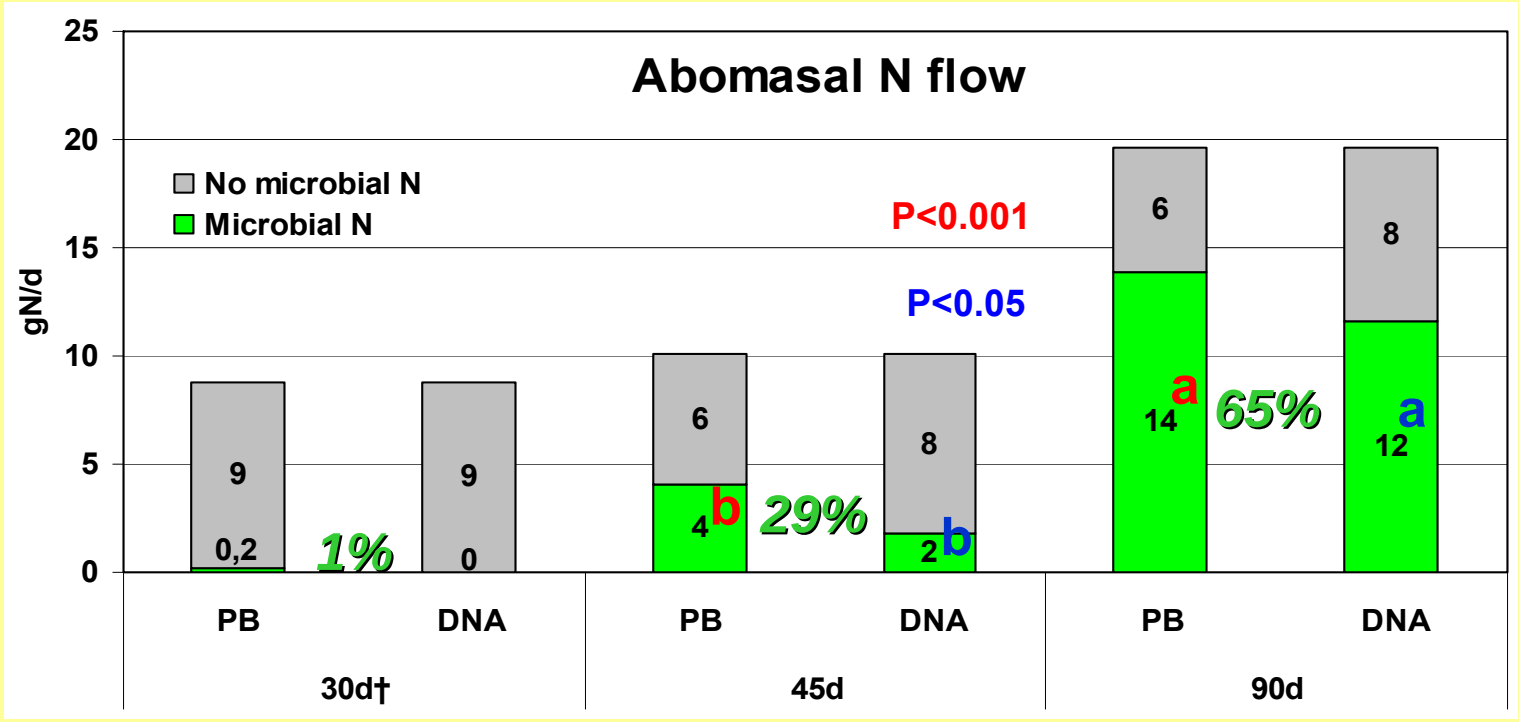
Microbial rumen development

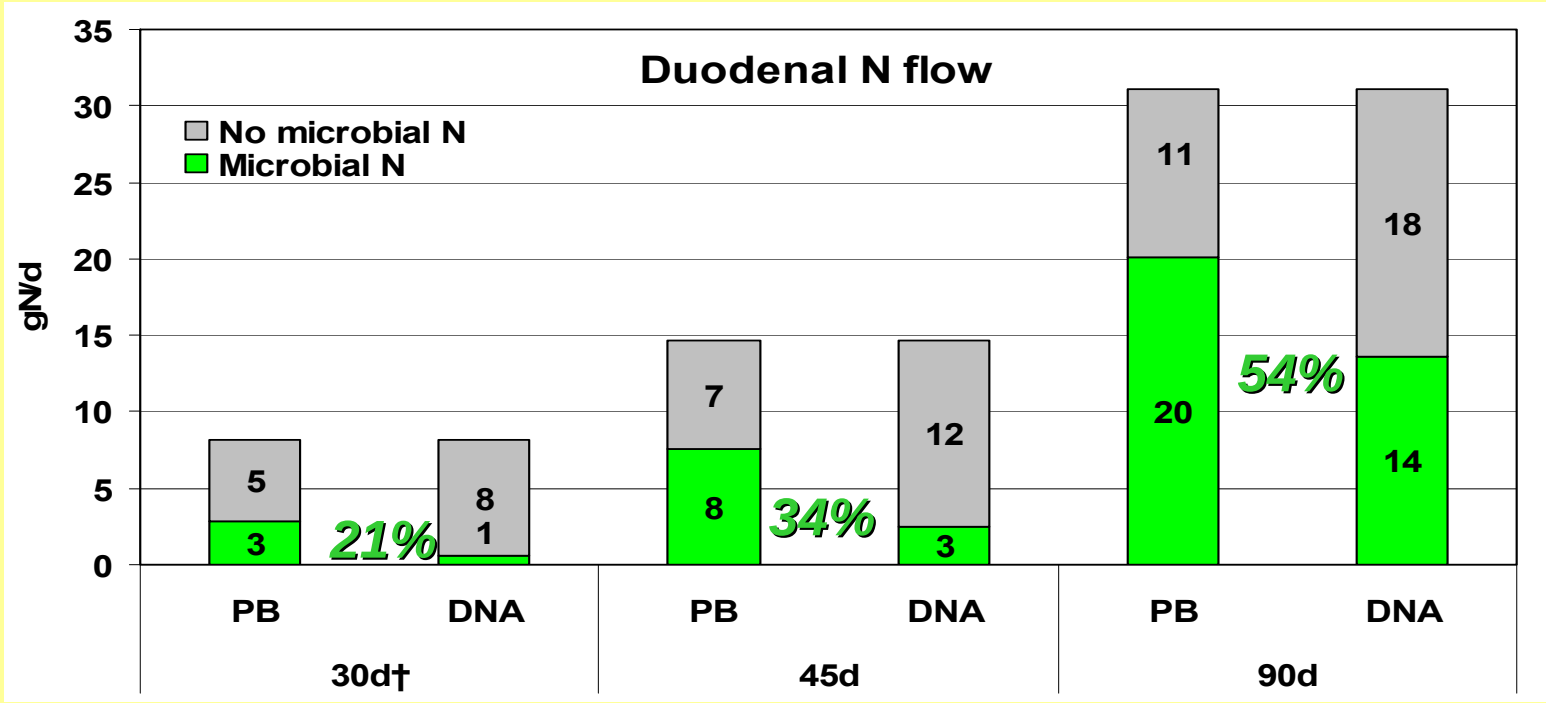




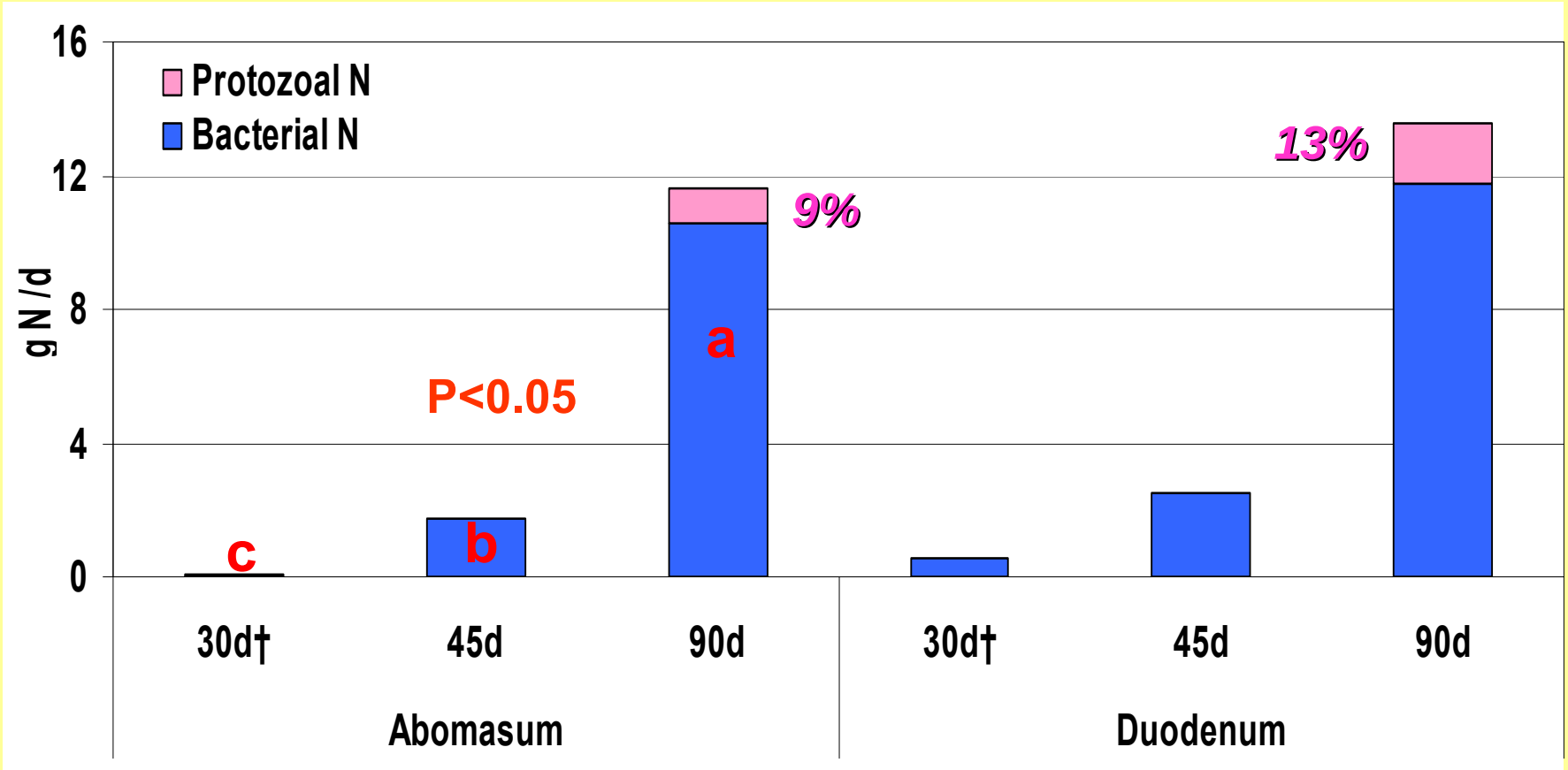


Microbial N flow

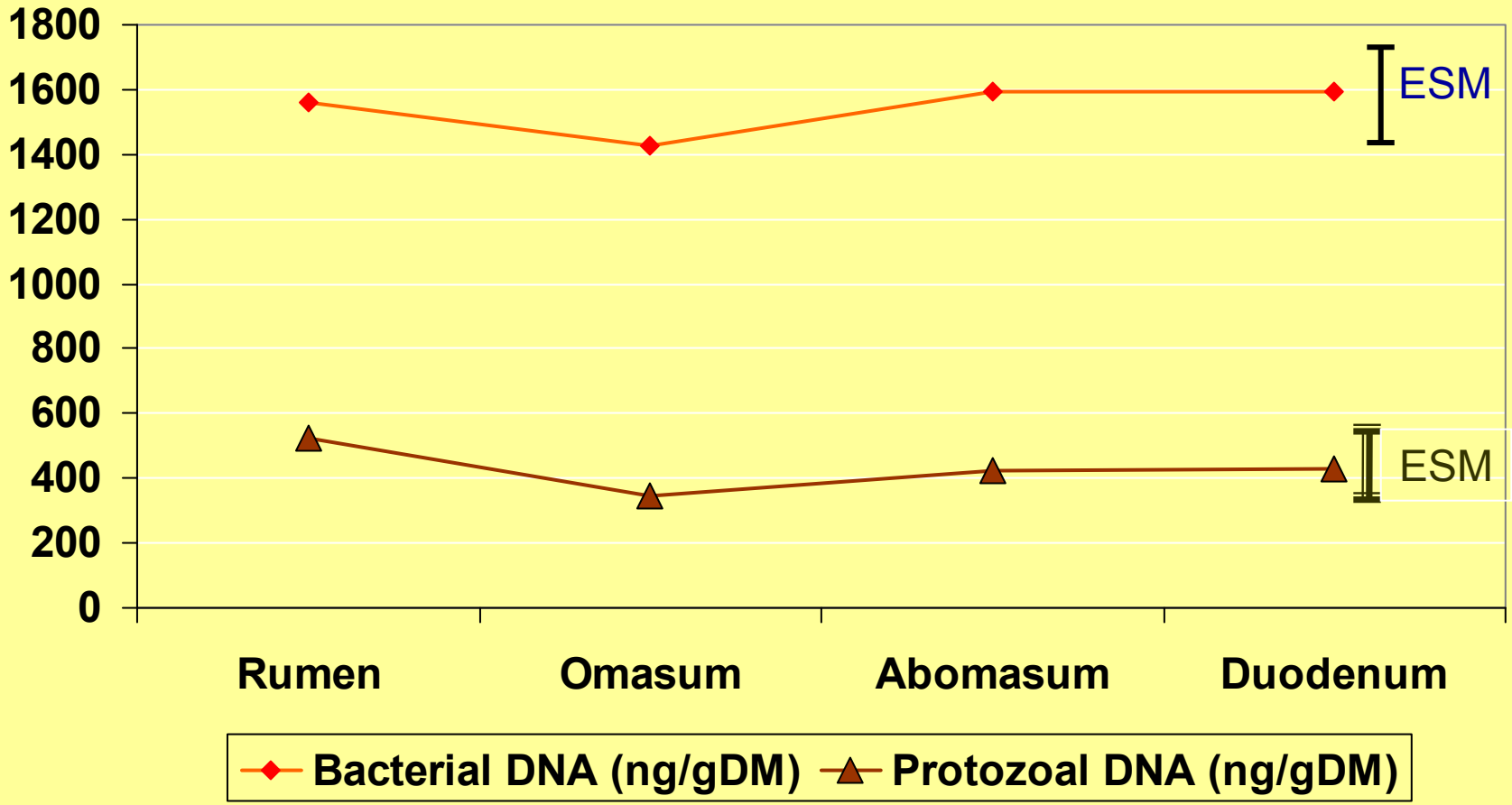




Origin of microbial N flow



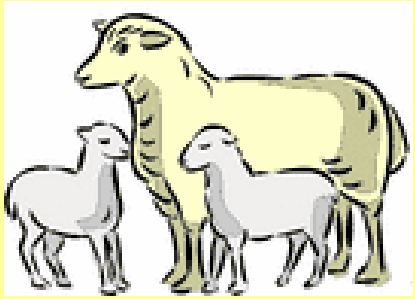
Evolution of DNA concentrations (ng/g dry matter) through digestive tract of lambs sacrificed at 90 days.



Implications

- **High proportion (up to 90 %) of Specific DNA-seq may survive gastric digestion**
- **Specific DNA-seq can be detected and then used as a specific microbial markers in either abomasal or duodenal digesta.**
- **Protozoa contribute significantly to duodenal digesta and its contributions depends on ciliate concentration into the rumen**

DNA-sequences behave like the conventional markers?



Objectives

- **To validate DNA-seq as a protozoa/bacterial marker comparing estimated values against those obtained using conventional markers as ^{15}N and purine bases (PB)**

- **To study the effect of protozoa and diet on rumen fermentation, digestibility and rumen protein synthesis**

Methodology

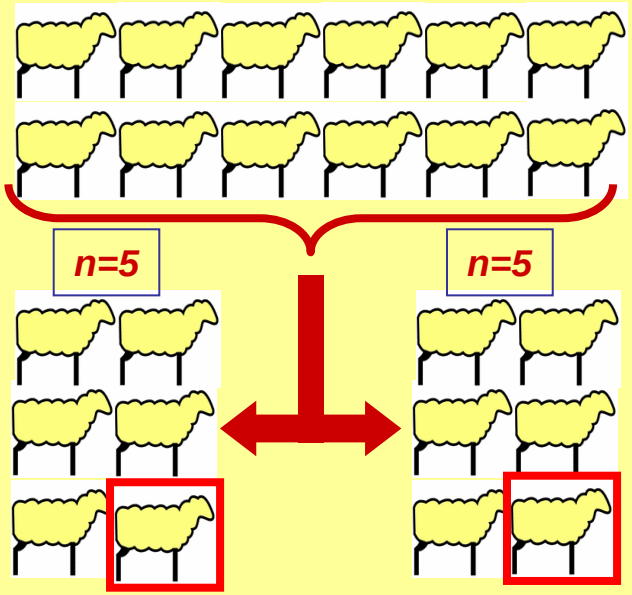
Weaning
45d

Fattening
Alfalfa hay
Compound feed

Beginning of assay
6 month

Animals and diets

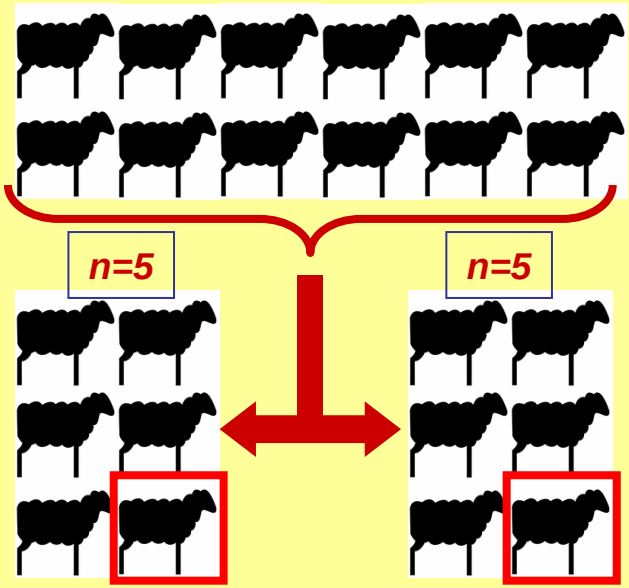
Control



100% Alfalfa

50% Alfalfa
50% Barley

Defaunated



100% Alfalfa

50% Alfalfa
50% Barley

3M+3F
Twins=Diet

1.5 maintenance
4 times/d

-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Diet adaptation

Adaptation to the cages

^{15}N

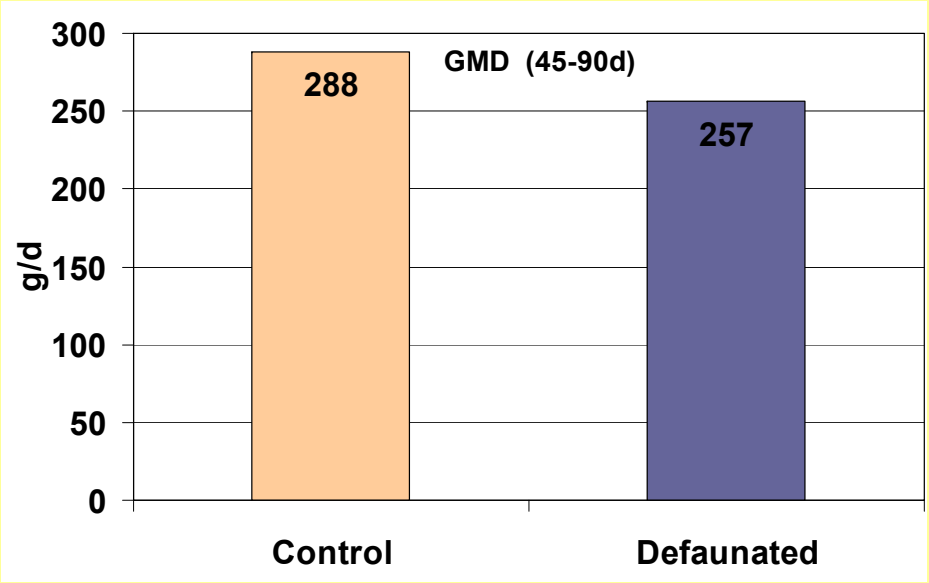
Digestibility balance

Get in cages

Slaughter

Sampling

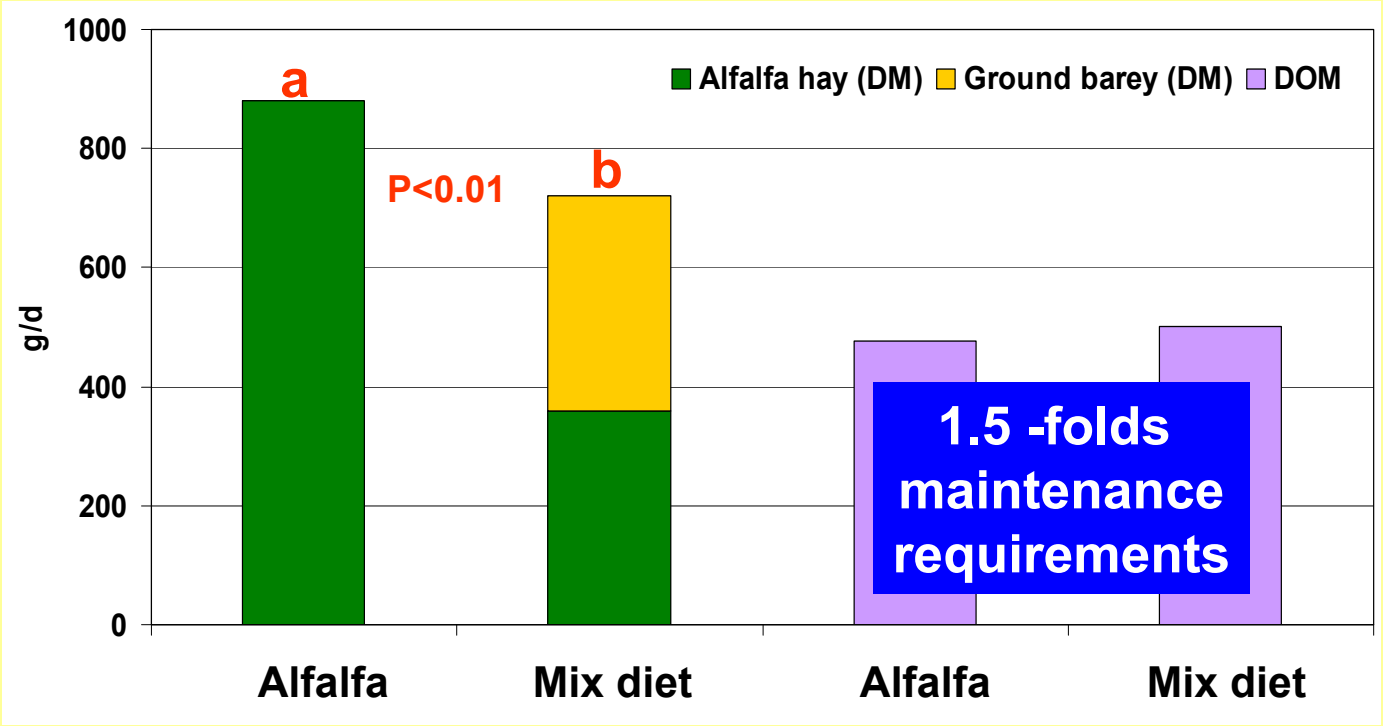
- **Rumen content**
  - Protozoal count — Microscopy — (Dehority, 1993; Lee *et al*., 1985)
  - VFA — GLC — (Jouany, 1982)
  - NH_3 — Spectrophotometry — (Chaney y Marbach, 1962)
- **Microbial extracts isolation**
  - Protozoa — Wash-filtration — (Sylvester *et al.,* 2004)
  - LAB — Centrifugation — (Pérez *et al*., 1998)
  - SAB — CMC y centrifugation — (Martín-Orúe *et al.,* 2000)
- **Abomasal flow** (*n*-alkanes C31)
  - Extraction — Saponification — (Mayes *et al.,* 1988; Oliván y Osoro 1999)
  - Analysis — GLC — (Valiente *et al*., 2003)
- **Microbial makers**
  - ^{15}N — Mass spectrophotometry
  - PB and PD — HPLC — (Balcells *et al*., 1992; Martín-Orúe *et al*., 1996)
  - DNA — Real time PCR a



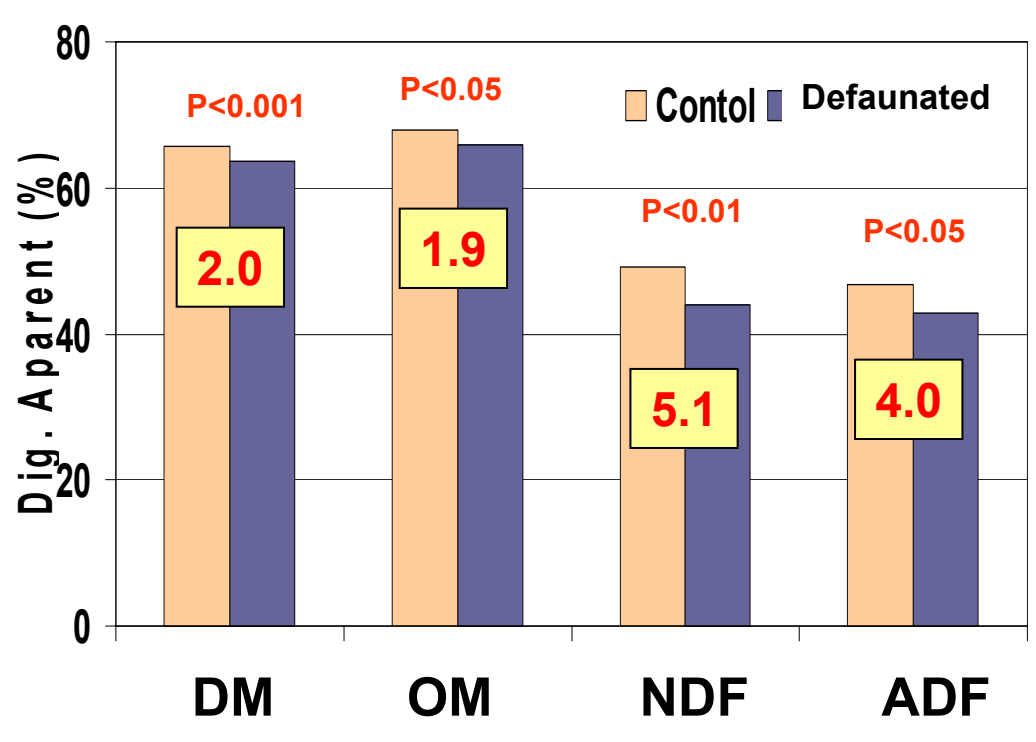
Results:

Growth

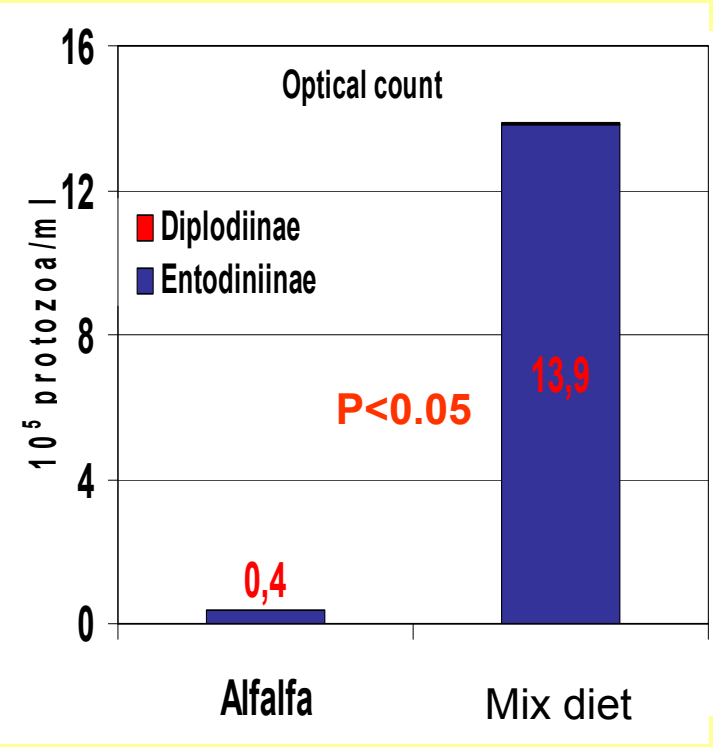
Intake

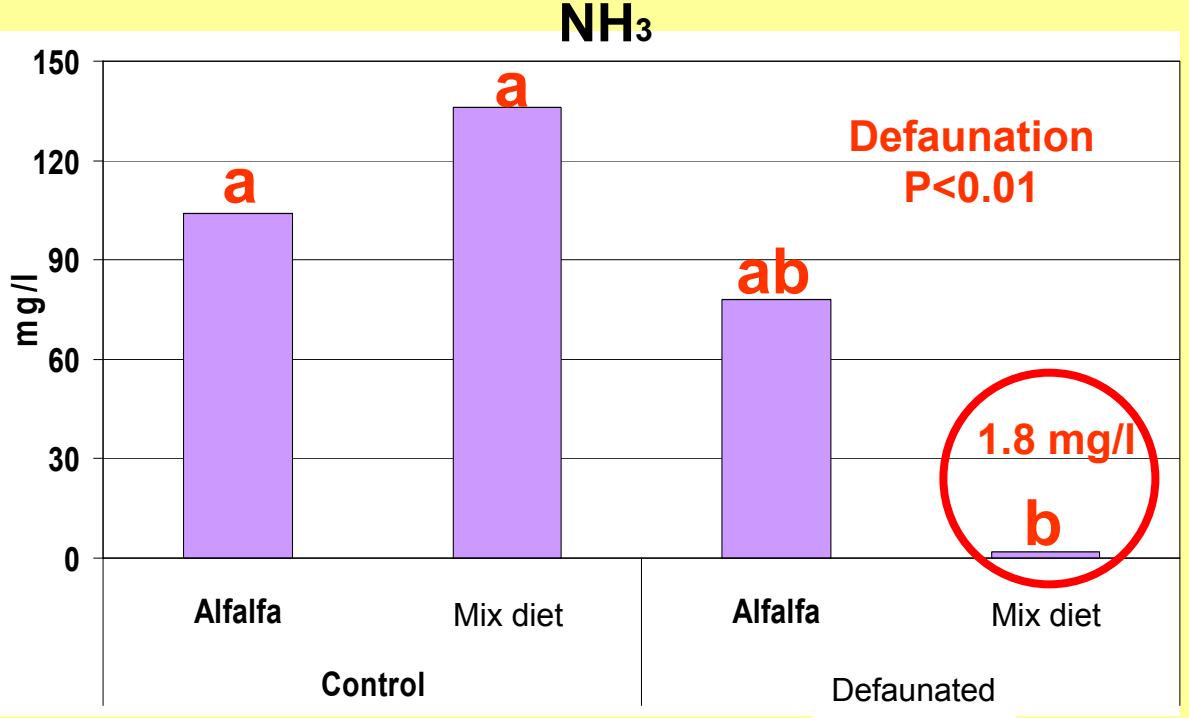


Effect of protozoa absence on fibre digestibility



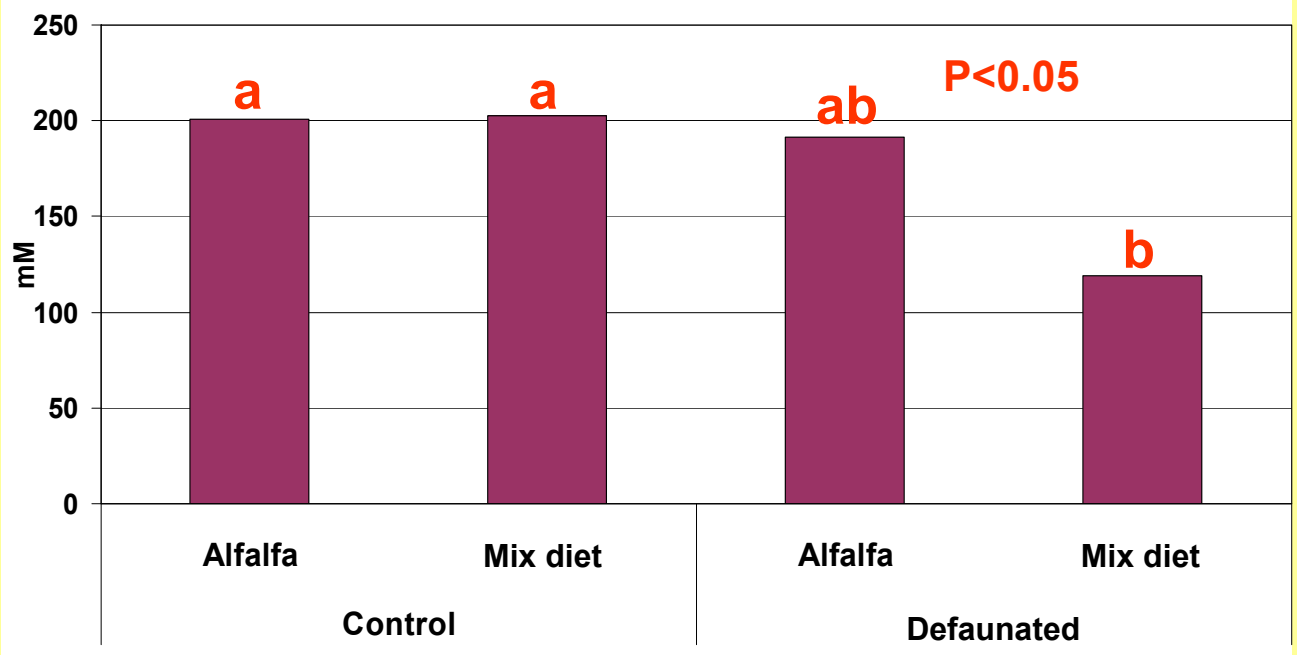
Rumen fermentation



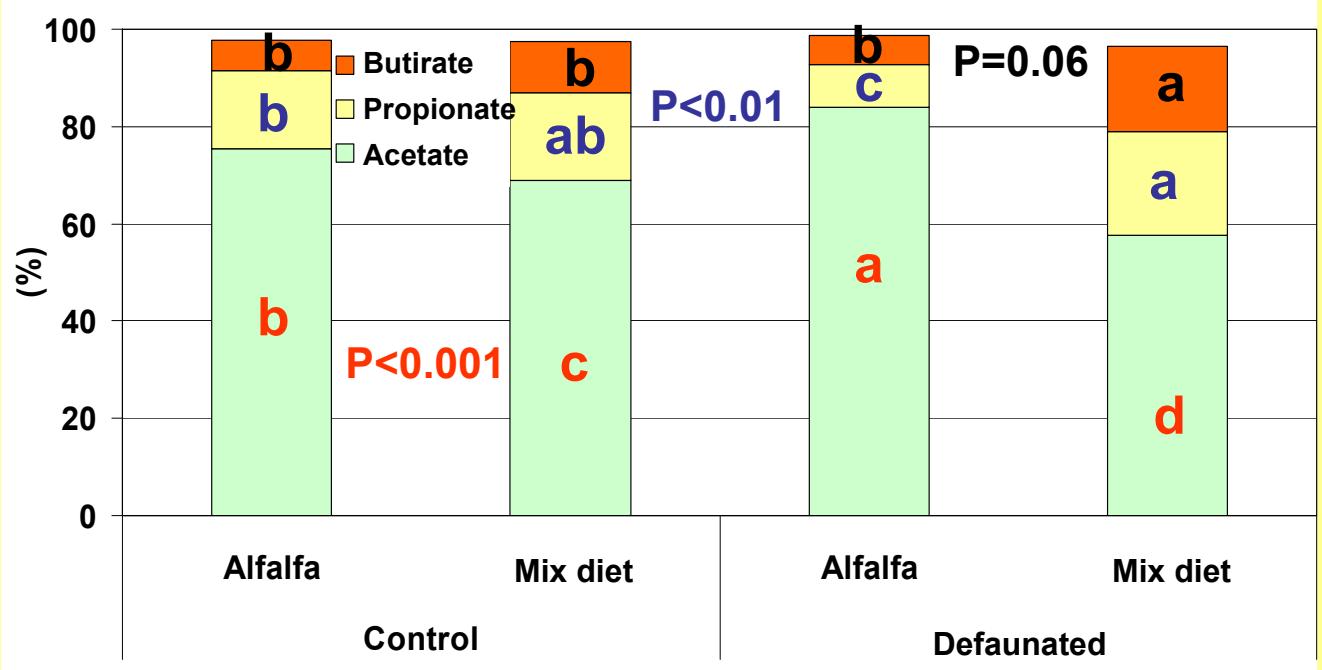


Fermentation products

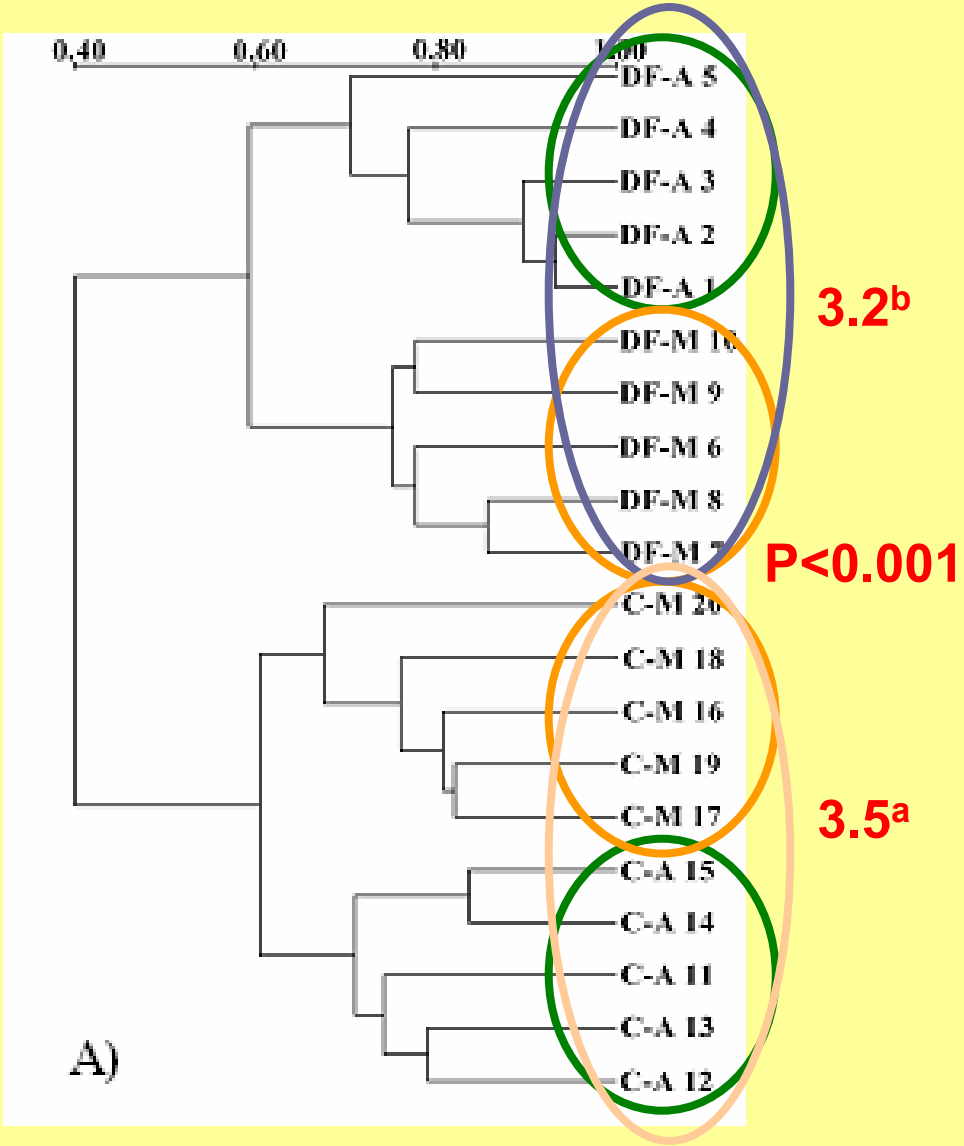
Total VFA

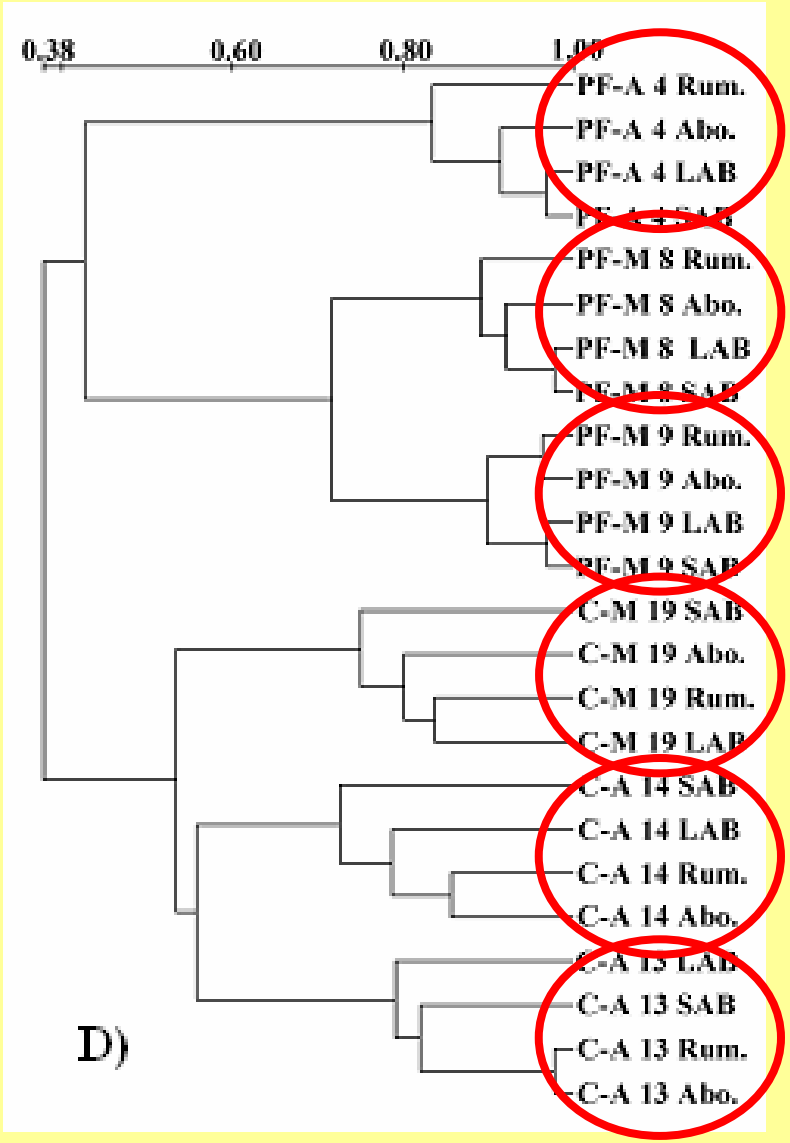


VFA proportion

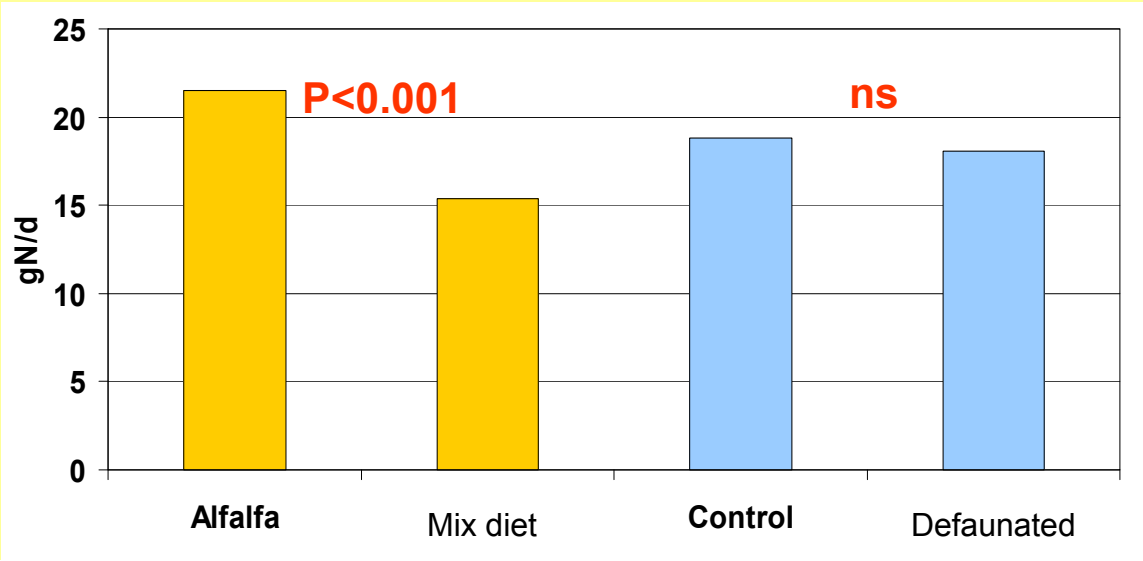


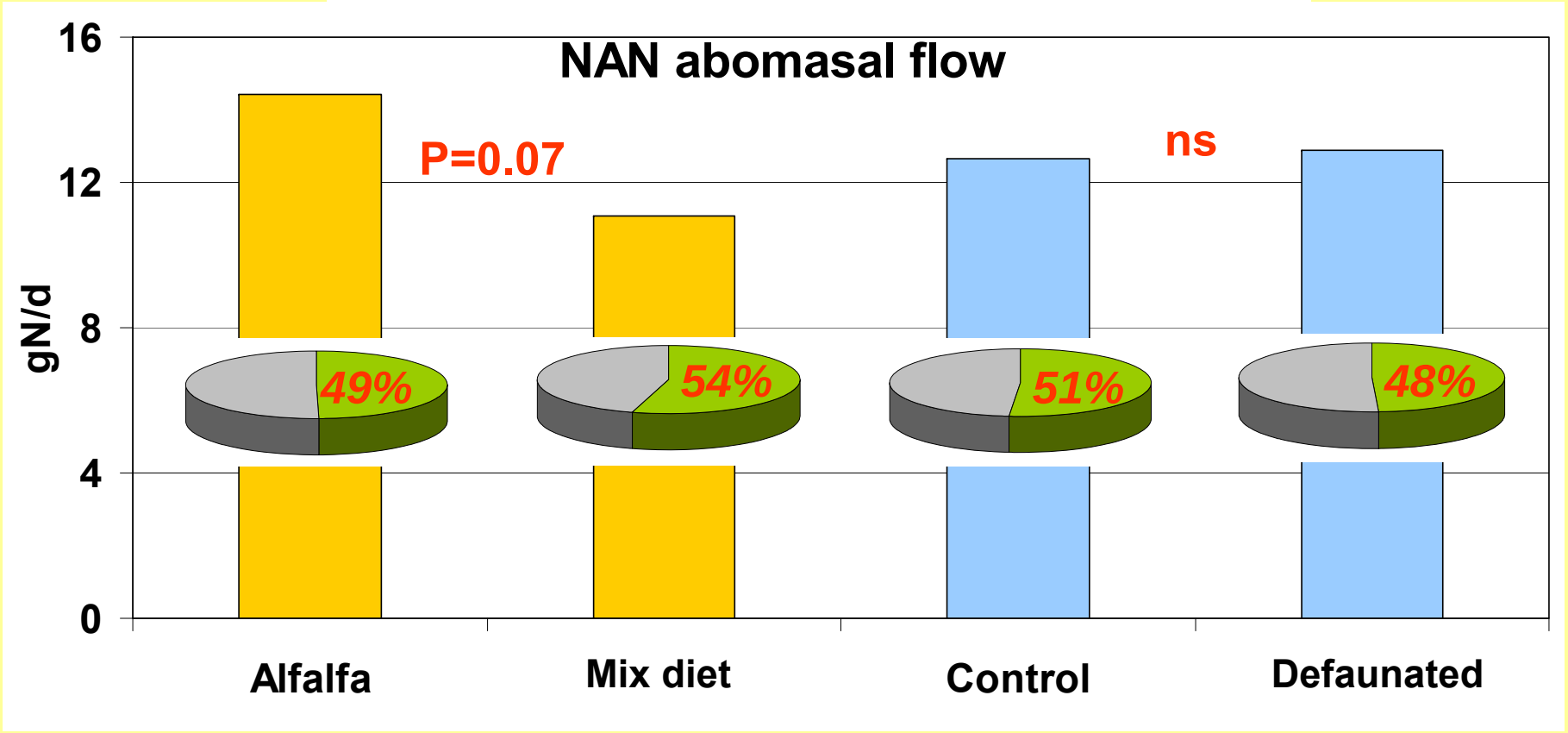
Bacterial diversity



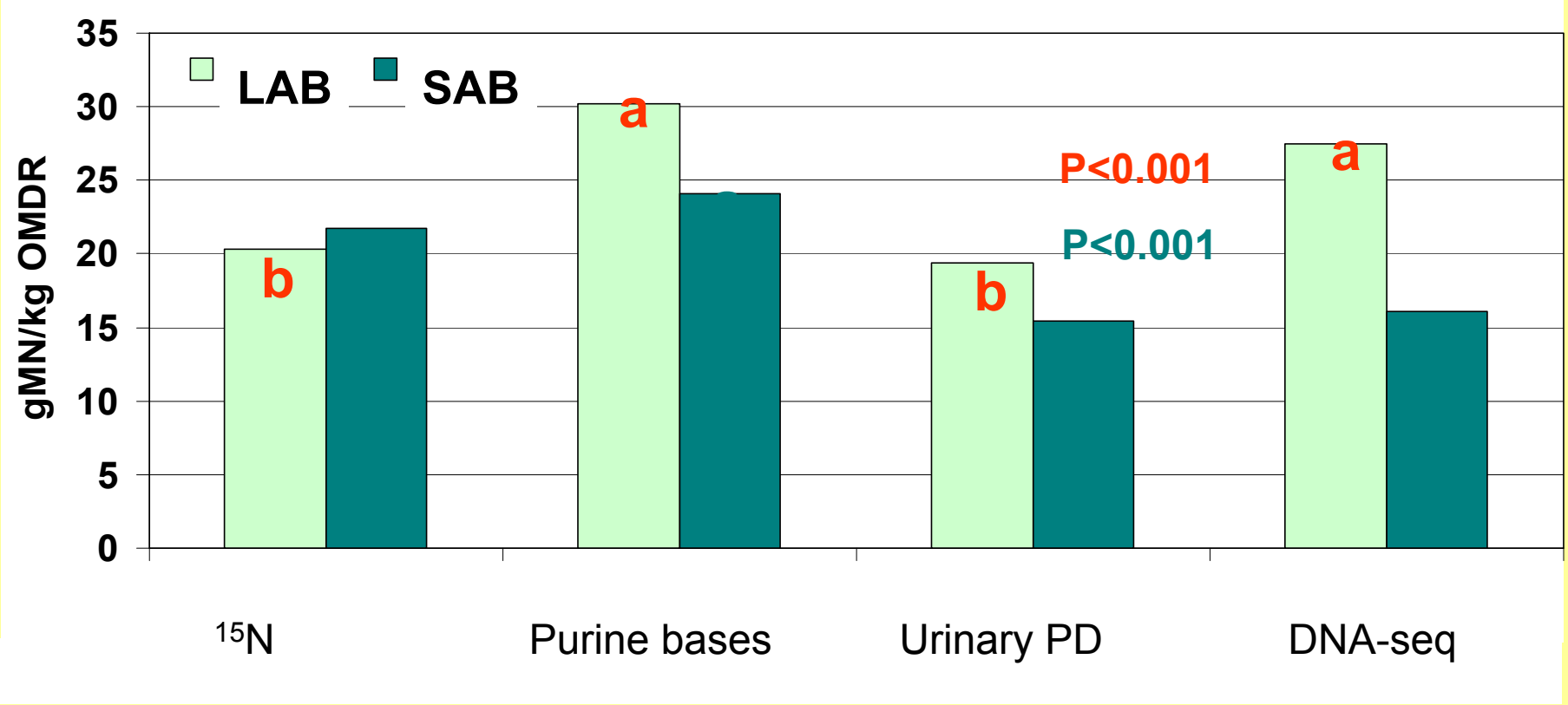


N intake


25
20
15
10
5
0
gN/d
P<0.001
ns
Alfalfa
Mix diet
Control
Defaunated

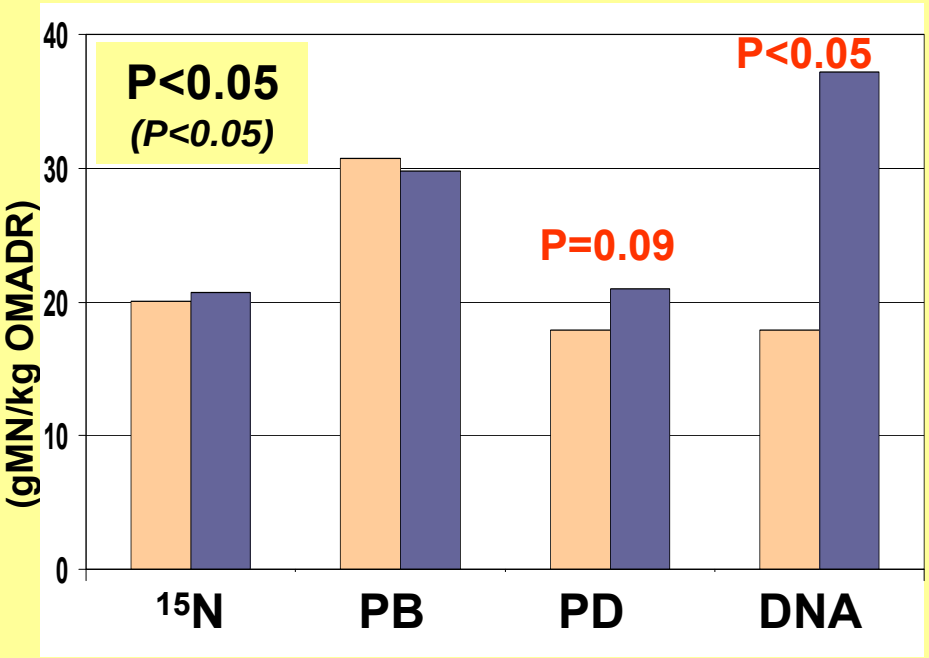

NAN abomasal flow
16
12
8
4
0
gN/d
P=0.07
ns
49%
54%
51%
48%
Alfalfa
Mix diet
Control
Defaunated

Effect of microbial extract and microbial marker on microbial synthesis efficiency (gMN/Kg OMDR)

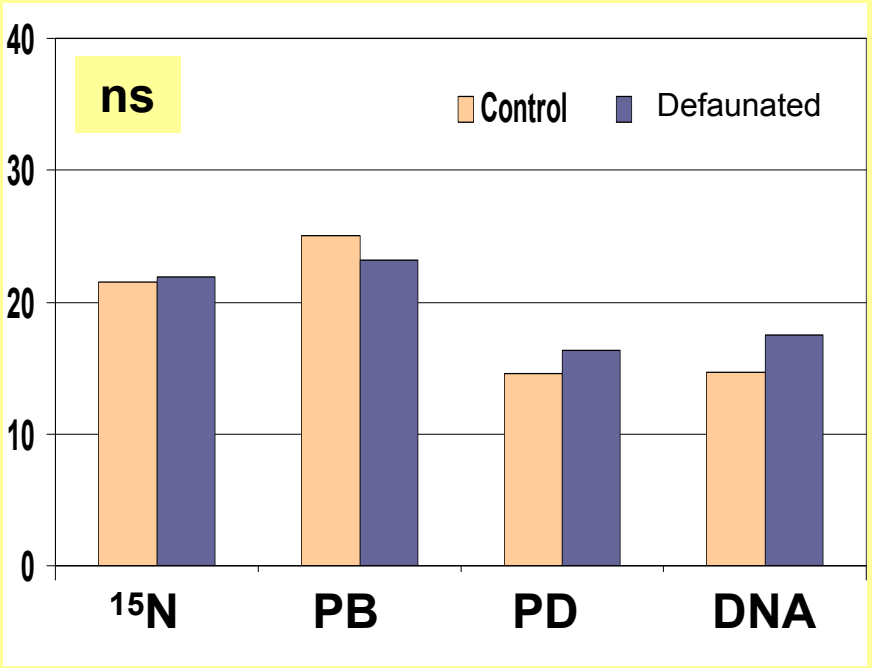


Effect of microbial marker and protozoa absence on microbial synthesis efficiency (gMN/Kg OMDR) using LAB or SAB as bacterial reference sample.

LAB



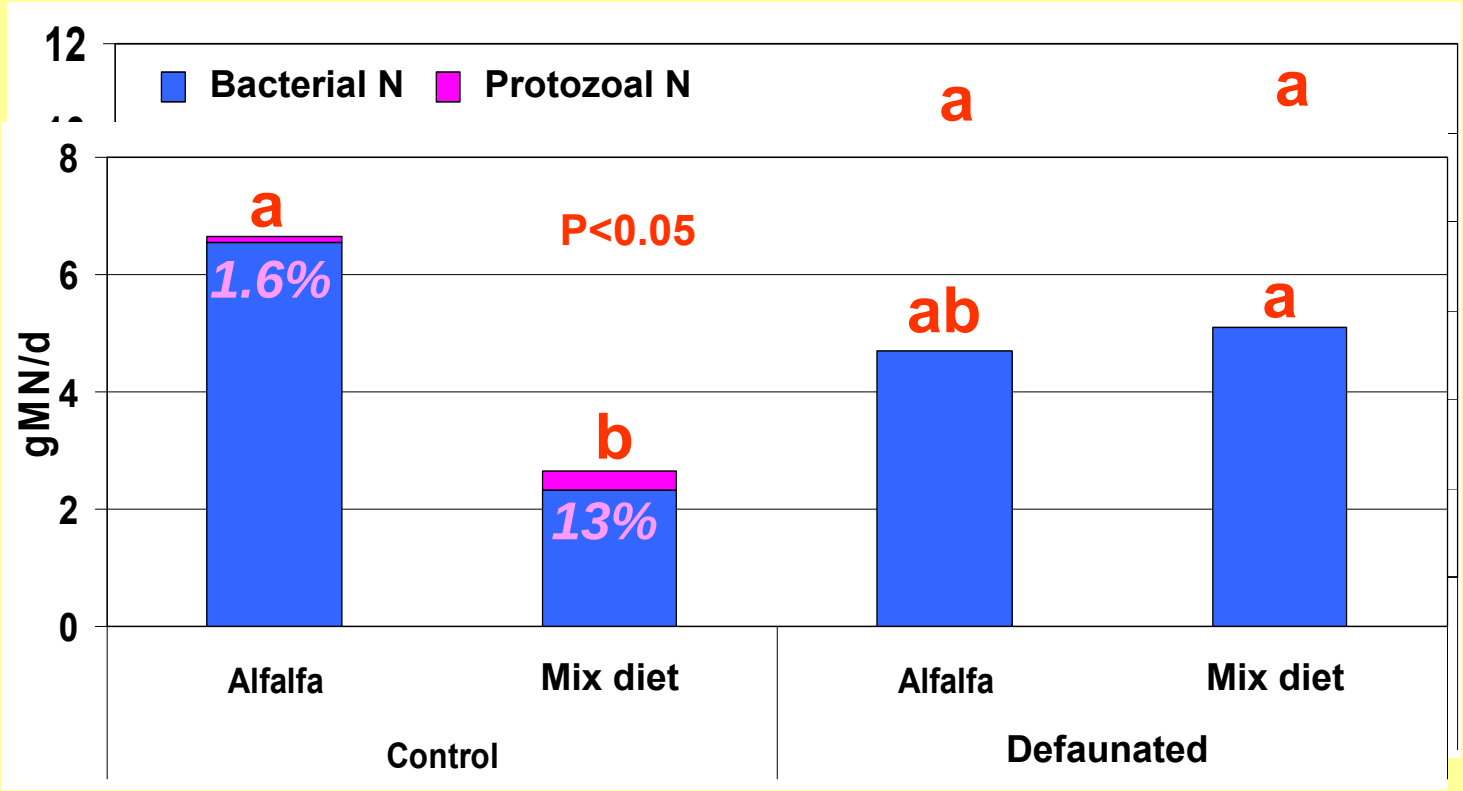
SAB



Using specific DNA-seq, protozoa-N contribution (g MN/day) to the duodenal digesta using two bacterial reference samples: LAB or SAB

LAB

SAB



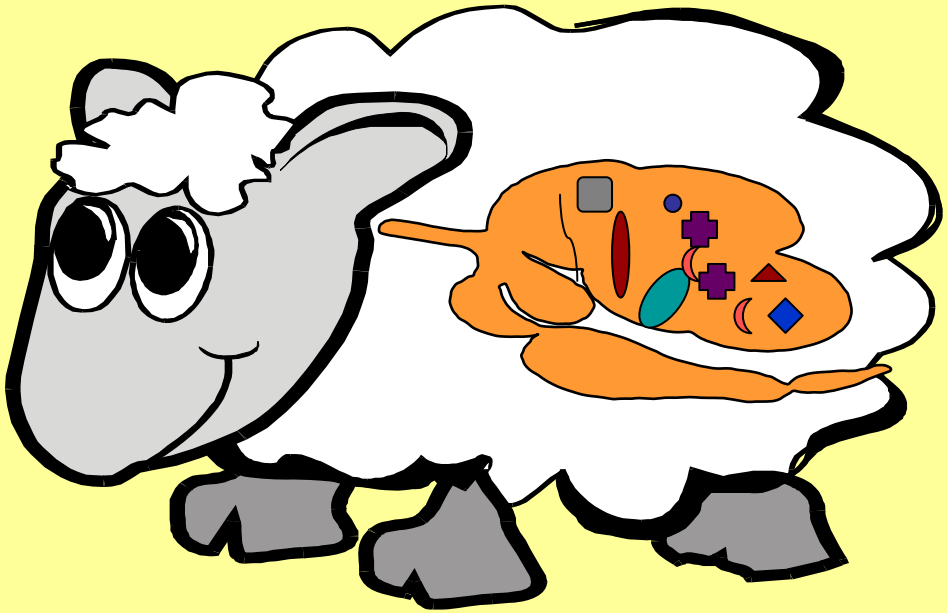
Implications

- **The absence of rumen protozoa alter rumen fermentation reducing digestibility, VFA production and rumen ammonia concentration.**

- **The effect of defaunation on microbial N flow and efficiency of microbial protein synthesis was weak and dependent of the type of marker and microbial reference used.**

- **The DNA-Seq was used successfully as a novel microbial marker and it allow the independent quantification of the bacterial and protozoa-N to duodenal digesta. It increased from 1.9 to 14.1% when alfalfa hay was supplemented with barley grain.**

- **More research should be need in this field to understand how the rumen protozoa can modify the rumen microbial ecosystem in general and the rumen N metabolism in particular.**



Thank you !!