PRIFYSGOL ABERYSTWYTH UNIVERSITY

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Institute of Biological, Environmental and Rural Sciences

Recent advances in identification of bacteria involved in rumen lipid metabolism

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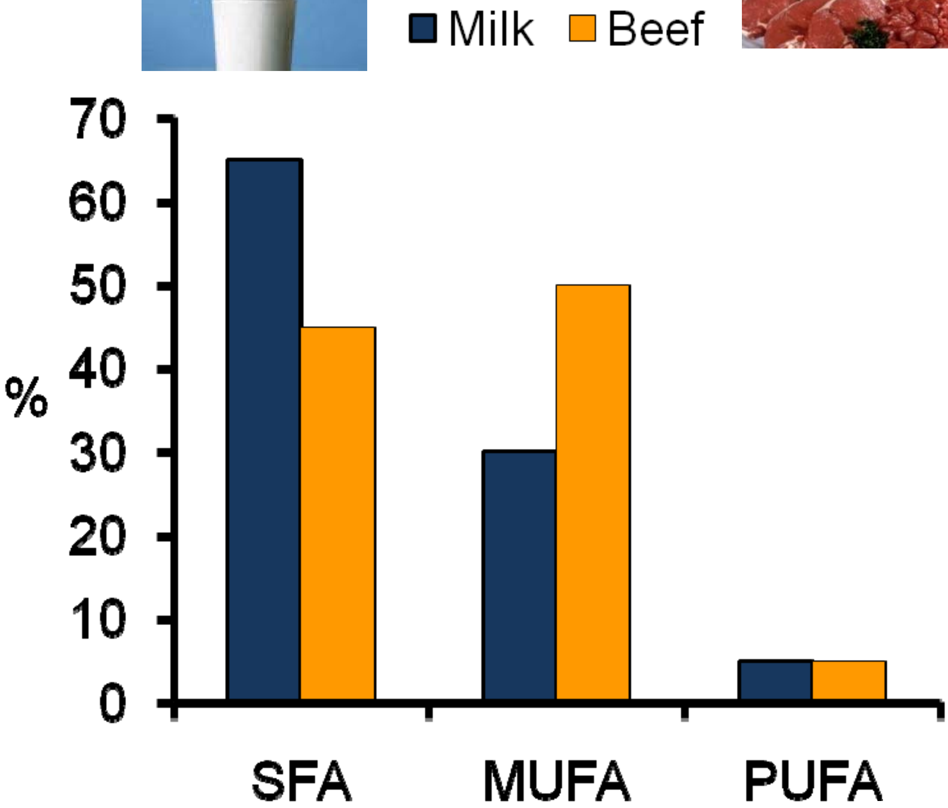
"LOOK, BUDDY... WE DON'T LIKE YOUR KIND IN HERE, OKAY?"

Outline

- Background
- Understanding of rumen microbial lipolysis
- Understanding of rumen microbial biohydrogenation
- Interactions of the rumen protozoa with the plant chloroplast
- Possible future directions
- Conclusions

"LOOK, BUDDY... WE DON'T LIKE YOUR KIND IN HERE, OKAY?"

Background: Fatty acids in ruminant products

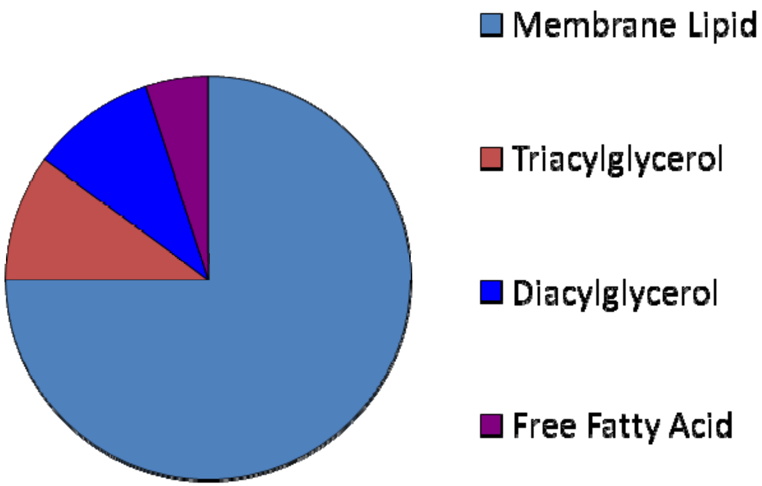


PUFA

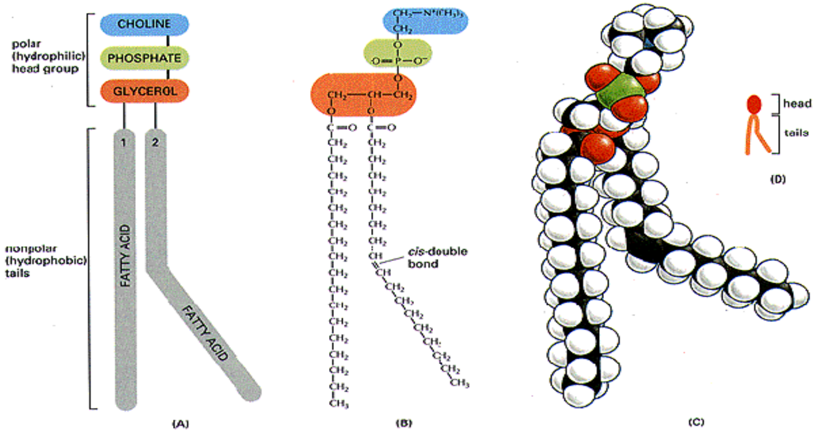
- linoleic acid (18:2*n*-6)
- α-linolenic acid (*18:3n-3*)
- eicosapentaenoic acid (20:5*n*-3; EPA)
- docosahexaenoic acid (22:6*n*-3; DHA)
- conjugated linoleic acids

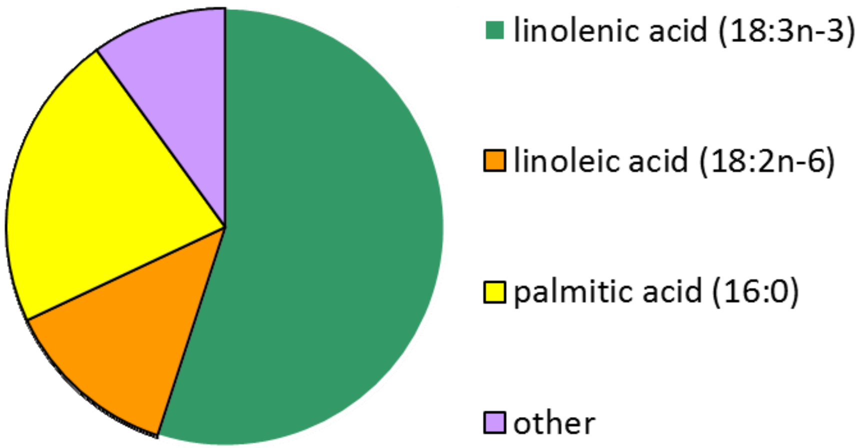
Need to improve healthiness of ruminant products

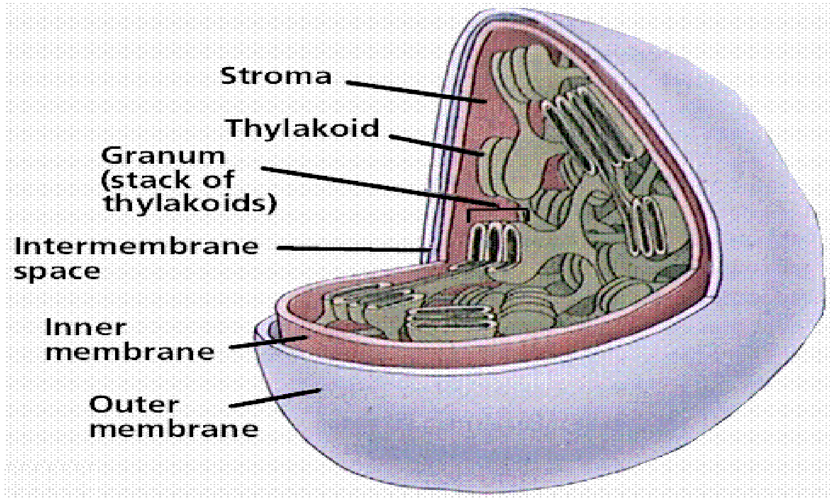
Perennial ryegrass lipids: opportunity



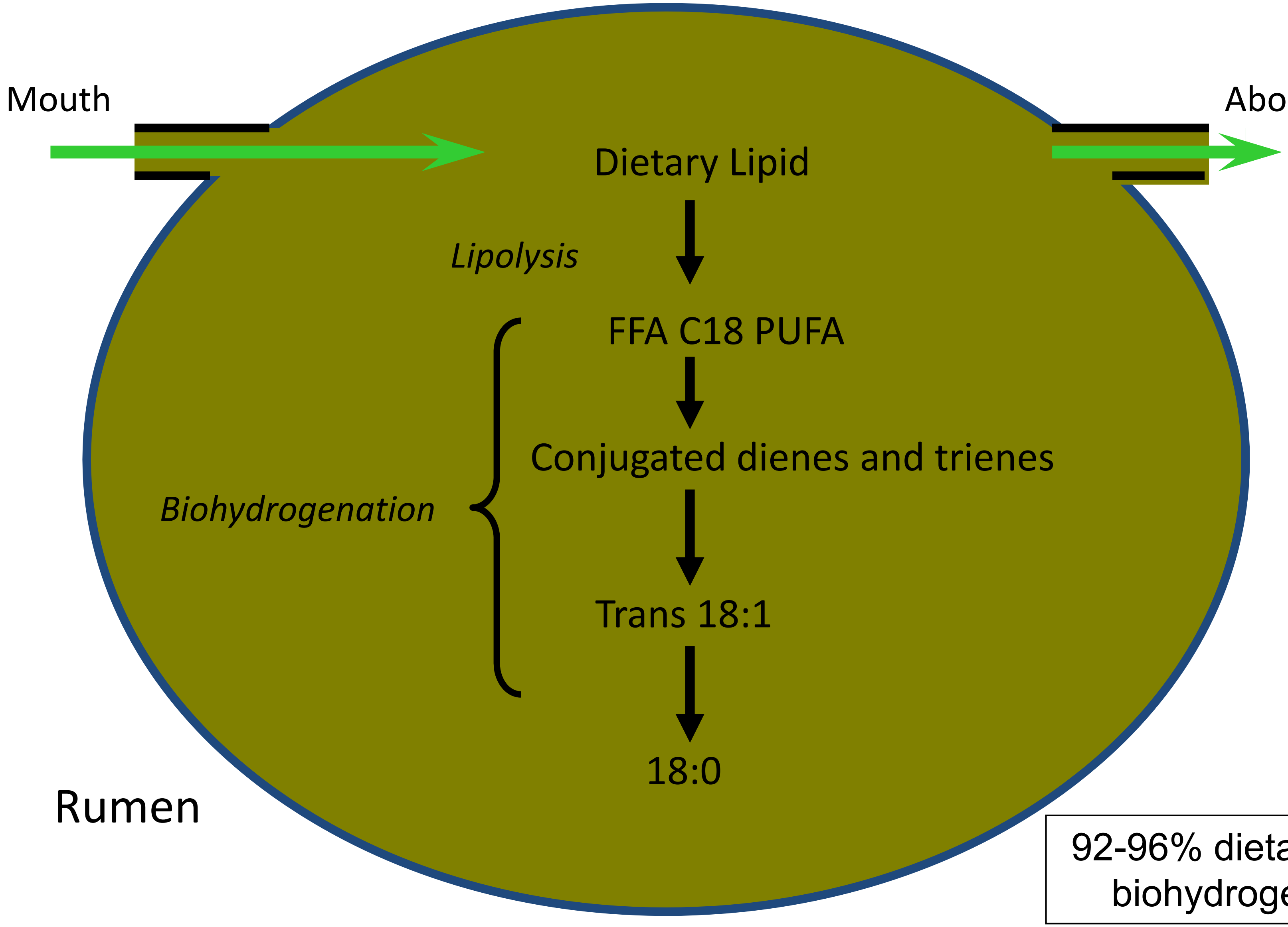
Three diagrams of the structure of phosphotidylcholine, a common phospholipid in membranes





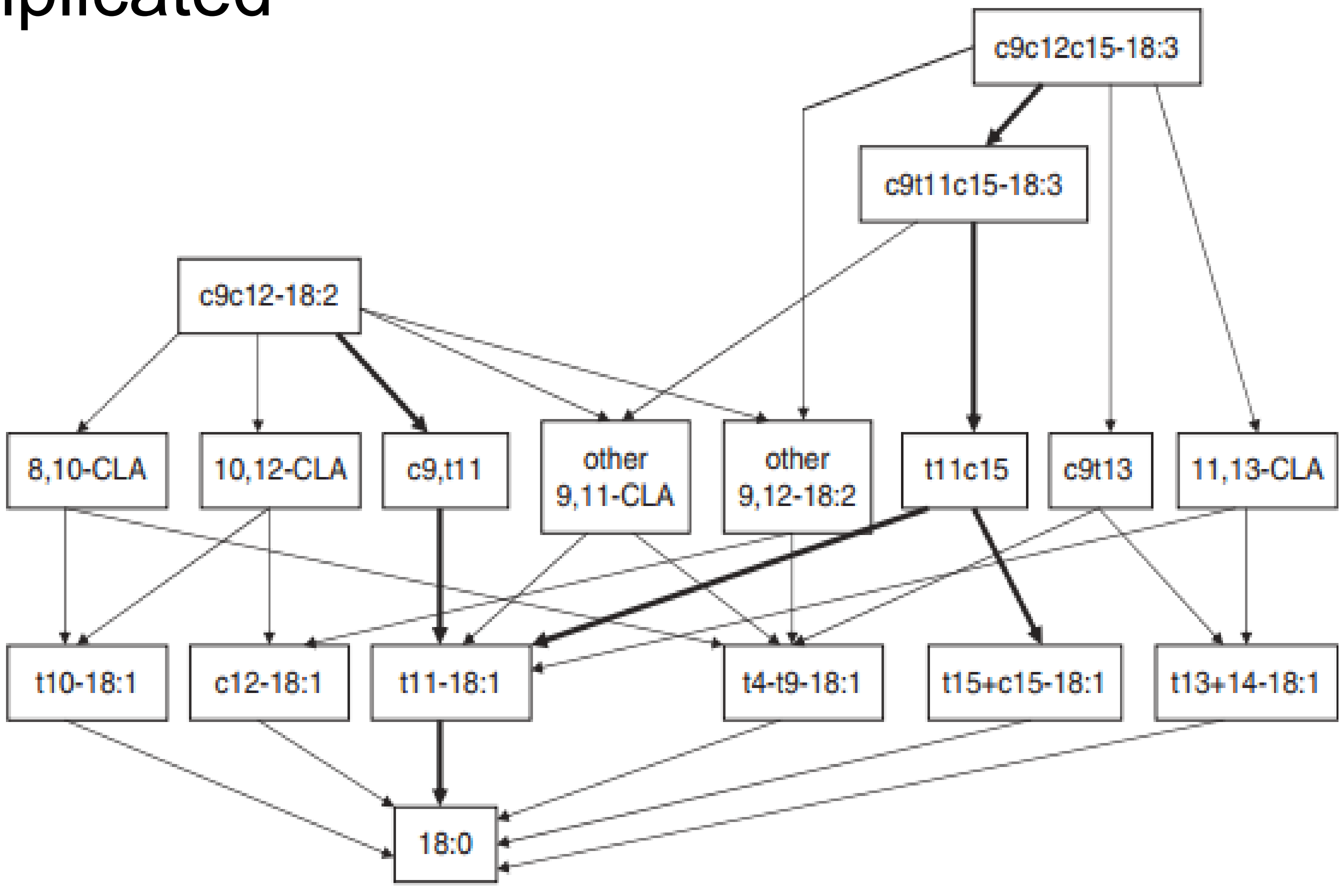


Overview of lipolysis and biohydrogenation within the rumen



92-96% dietary PUFA biohydrogenated

Biohydrogenation pathways are more complicated



Now know other triene intermediates, such as 9,11,13; cis-7, cis-12, cis-15; cis-8, cis-12, cis-15 are formed from LNA (Honkanen *et al*., 2016)

Taken from Chilliard *et al*., 2006

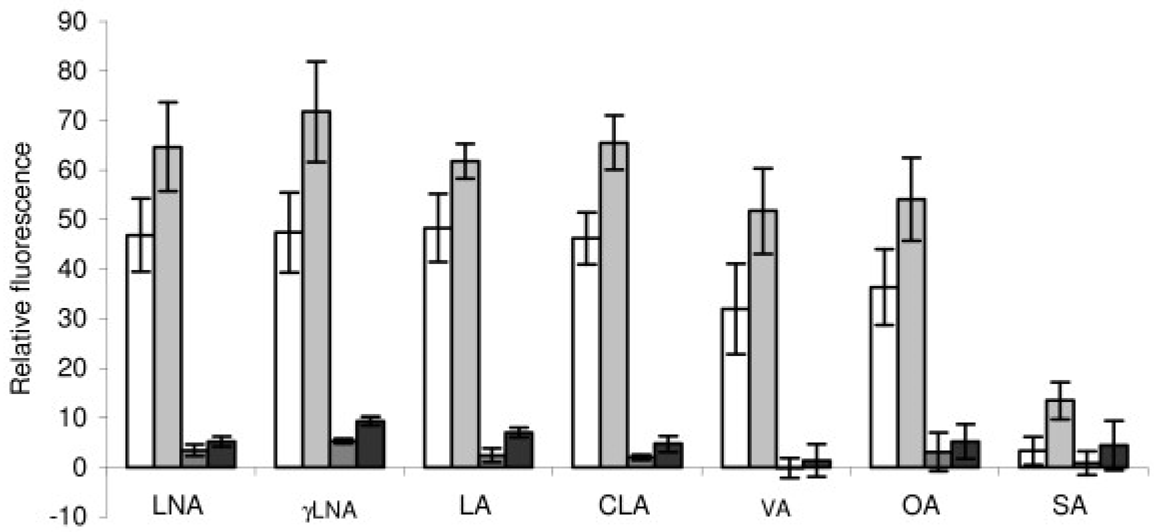
Maia *et al. BMC Microbiology* 2010, **10**:52
http://www.biomedcentral.com/1471-2180/10/52

BMC Microbiology

RESEARCH ARTICLE **Open Access**

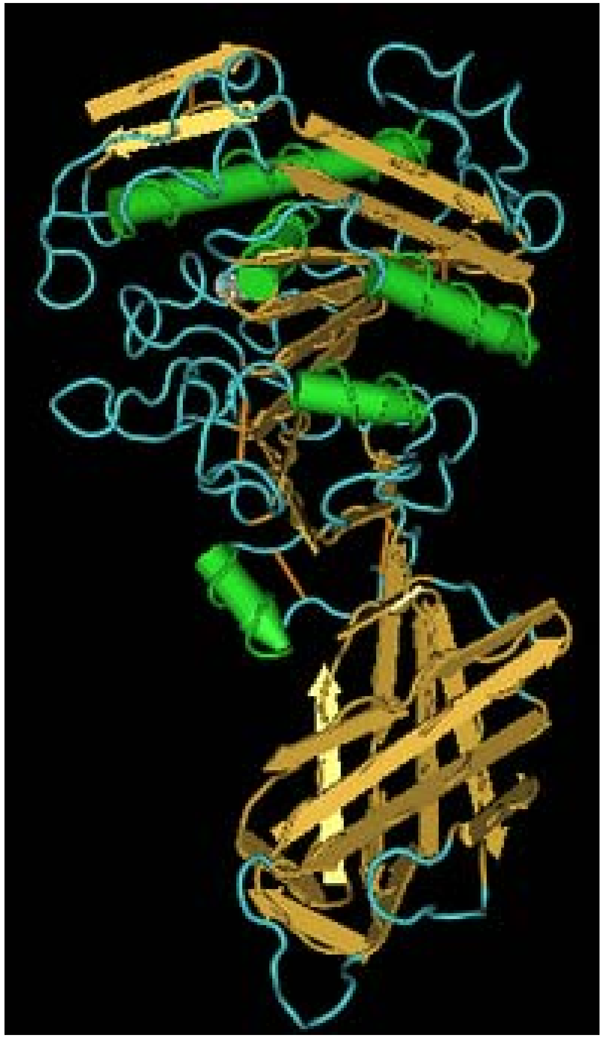
Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*

Margarida RG Maia[1,2,4], Lal C Chaudhary[1,5], Charles S Bestwick[1], Anthony J Richardson[1], Nest McKain[1], Tony R Larson[3], Ian A Graham[3], Robert J Wallace[1*]

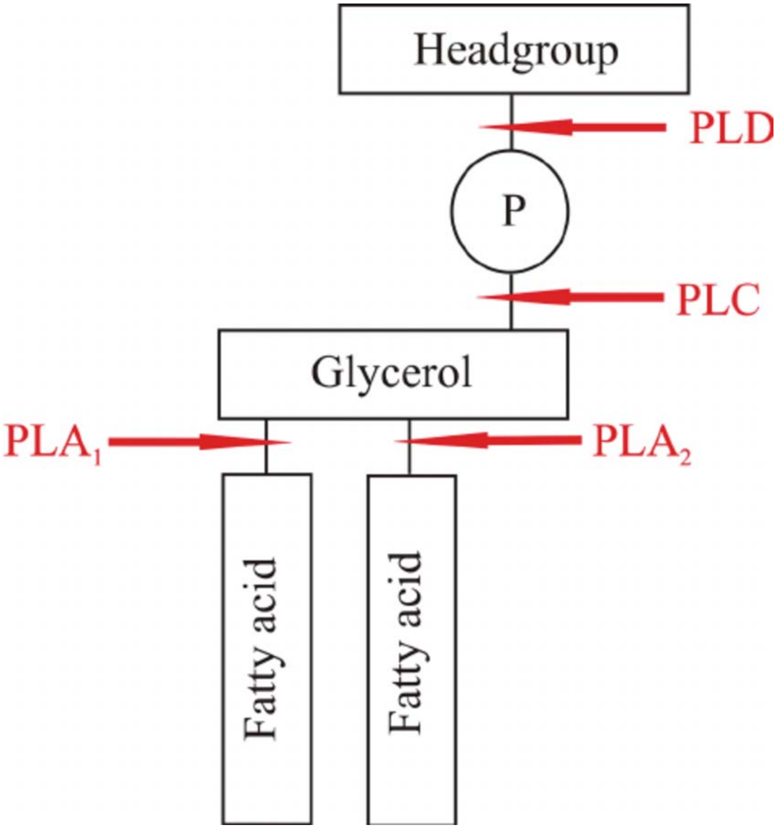


Fatty acid methyl esters reduces the integrity of *Butyrivibrio fibrosolvens* JW11

Understanding of rumen microbial lipolysis

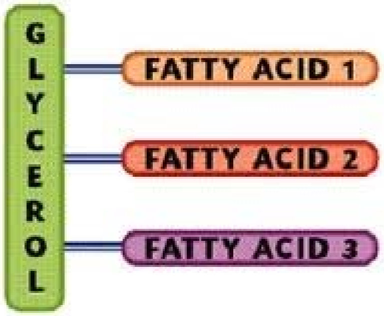


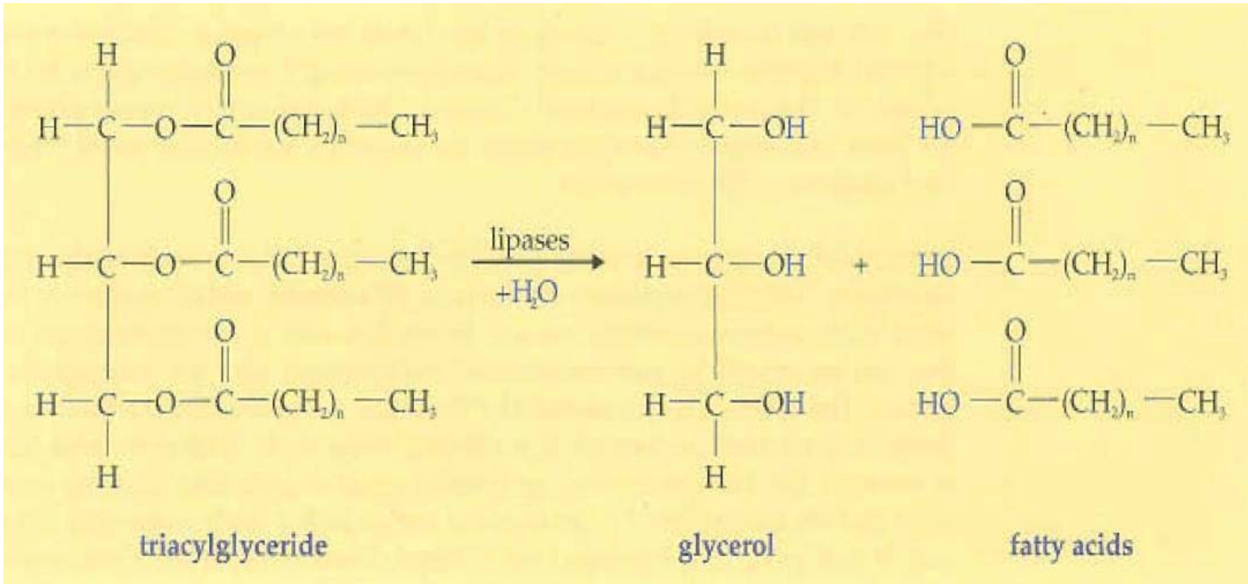
Lipolysis of phospholipids (membrane lipids)



PLD: Phospholipase D; **PLC:** Phospholipase C; **PLA$_1$:** Phospholipase A$_1$; **PLA$_2$:** Phospholipase A$_2$. All are present in perennial ryegrass and many of the rumen microbiota, in particular PLA$_1$ and PLA$_2$.

Lipolysis of triacylglycerols





Triacylglycerols are found mainly in oils

Understanding of rumen lipolysis

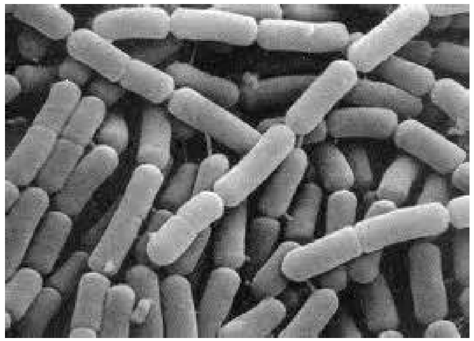
- Plant undergo lipolysis under abiotic and biotic stress
- Faruque *et al.* (1974) concluded that plant lipases contributed to more release of non-esterified fatty acids than bacterial lipases in the rumen.
- Others suggest bacterial lipases more important e.g Dawson *et al*. (1977).
- No real consensus to date

Rumen bacterial lipolysis

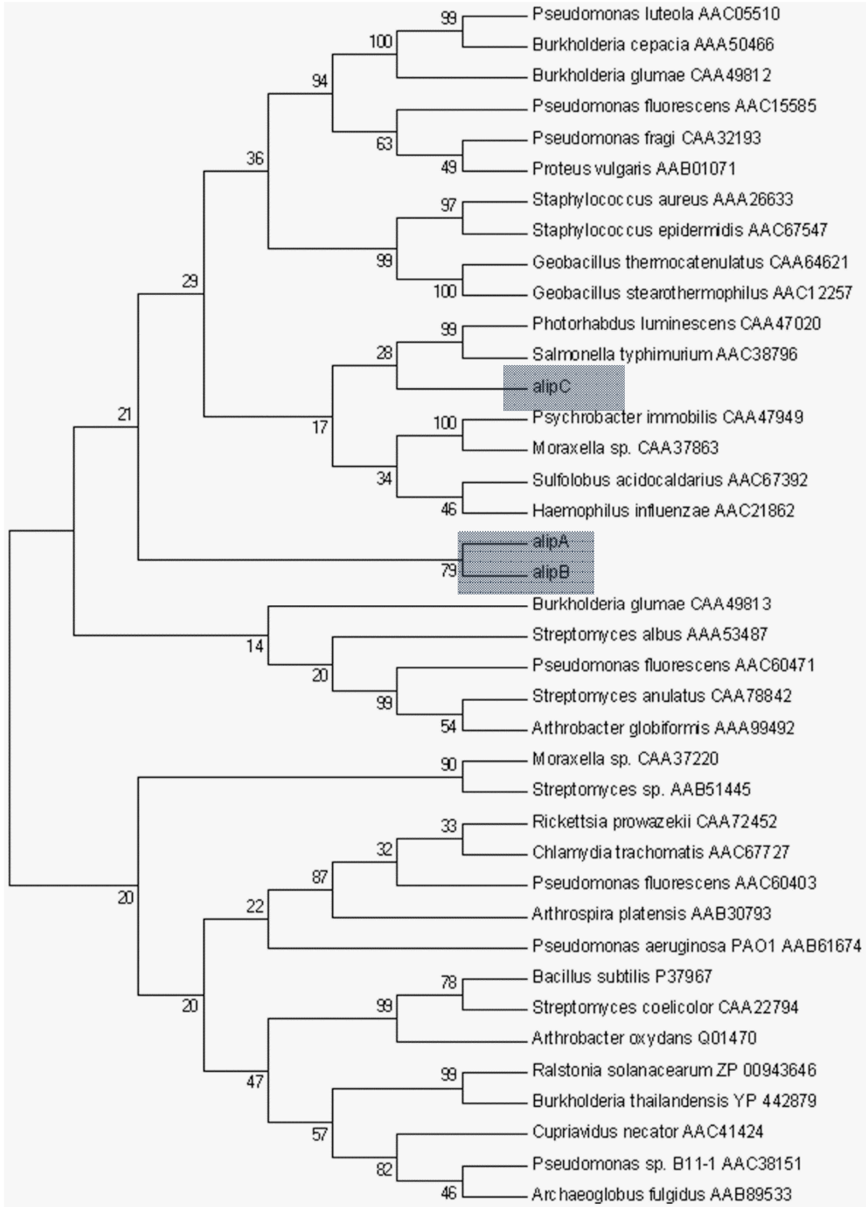
- Historically only *Anaerovibrio lipolyticus* and *Butyrivibrio* spp. implicated.

- *A. lipolyticus* known to hydrolyse triglycerides to glycerol and non-esterified fatty acids, but thought unlikely to be able to hydrolyse phospholipids.

- *Butyrivbrio* spp. able to hydrolyse phospholipids.



A. lipolyticus draft genome sequence revealed the possession of family II and V lipases



Prive *et al*., 2013. Identification and characterization of three novel lipases belonging to families II and V from *Anaerovibrio lipolyticus* 5ST. *PLOS ONE*, e69076

A. lipolyticus lipase specificity

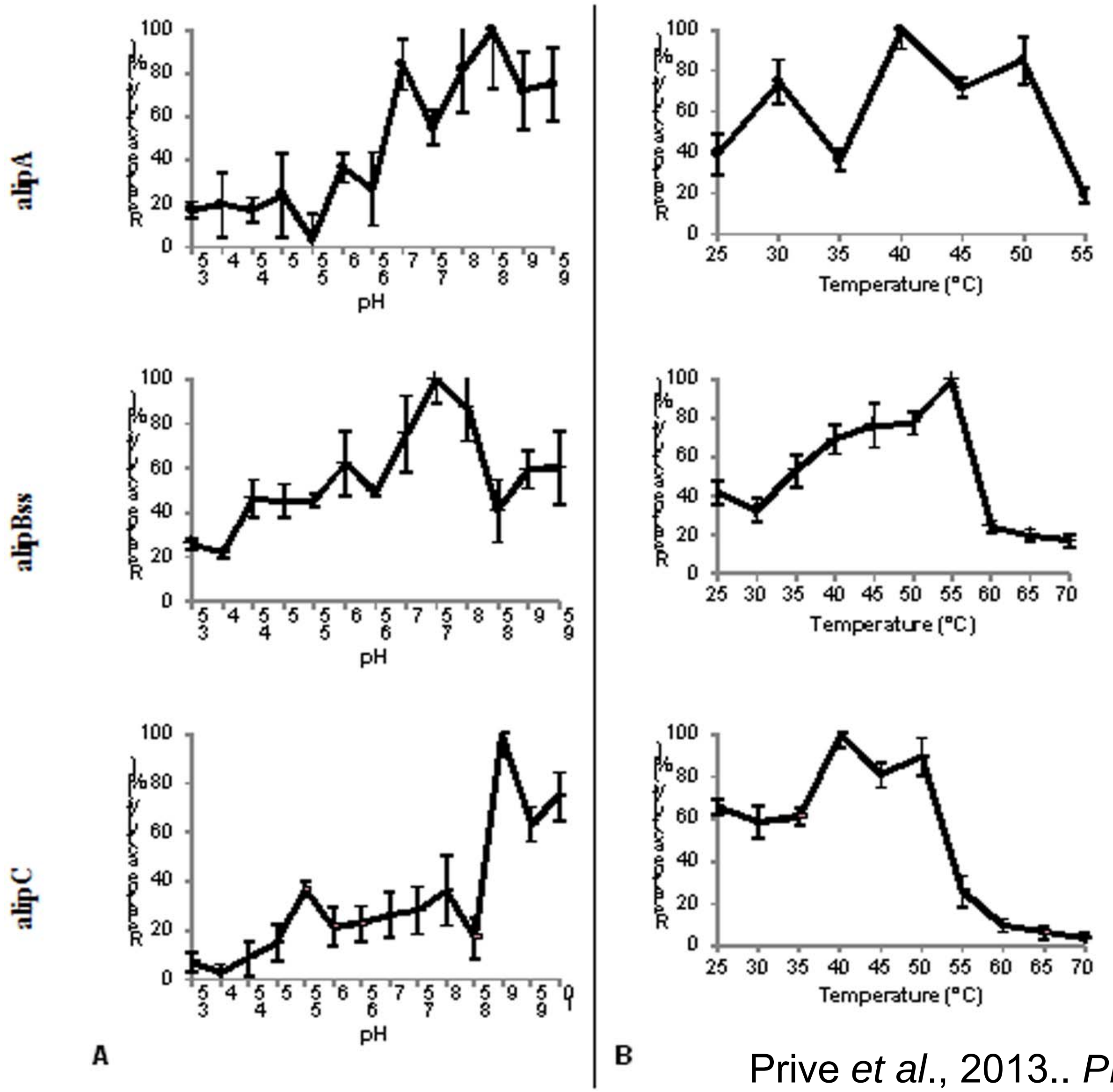
Substrate	Specific activity (U·mg^{-1} protein)		
	alipA	alipBss	alipC
pNP-acylesters			
Butyrate (C4)	139.4±17.4	91.8±22.9	186.7±41.5
Caproate (C6)	73.8±19.0	48.6±11.5	76.0±48.9
Caprylate (C8)	98.4±17.4	59.4±21.8	269.6±71.7
Caprate (C10)	32.8±9.5	43.2±11.4	117.5±5.3
Laurate (C12)	639.6±24.0	97.2±24.7	242.0±38.4
Myristate (C14)	172.2±17.4	156.6±11.5	193.6±34.2
Palmitate (C16)	123.0±19.1	64.8±27.6	83.0±37.5
Stearate (C18)	73.8±17.3	ND	34.6±20.8

ND. Not detected.
doi:10.1371/journal.pone.0069076.t004

AlipB and AlipC also showed activity against the triglycerides Tributyrin (C4), Tricaprylin (C8) and Triolein (C18:1)

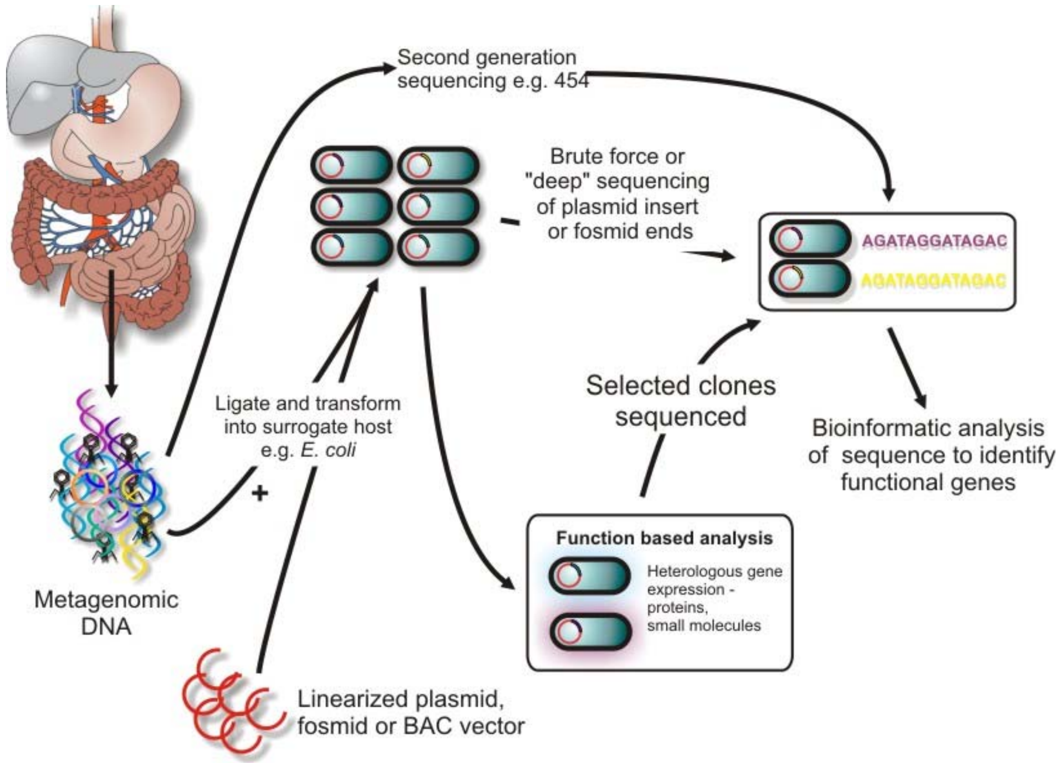
Prive *et al*., 2013.. *PLOS ONE*, e69076

A. lipolyticus isolated lipases show pH and temperature tolerance



Prive *et al*., 2013.. *PLOS ONE*, e69076

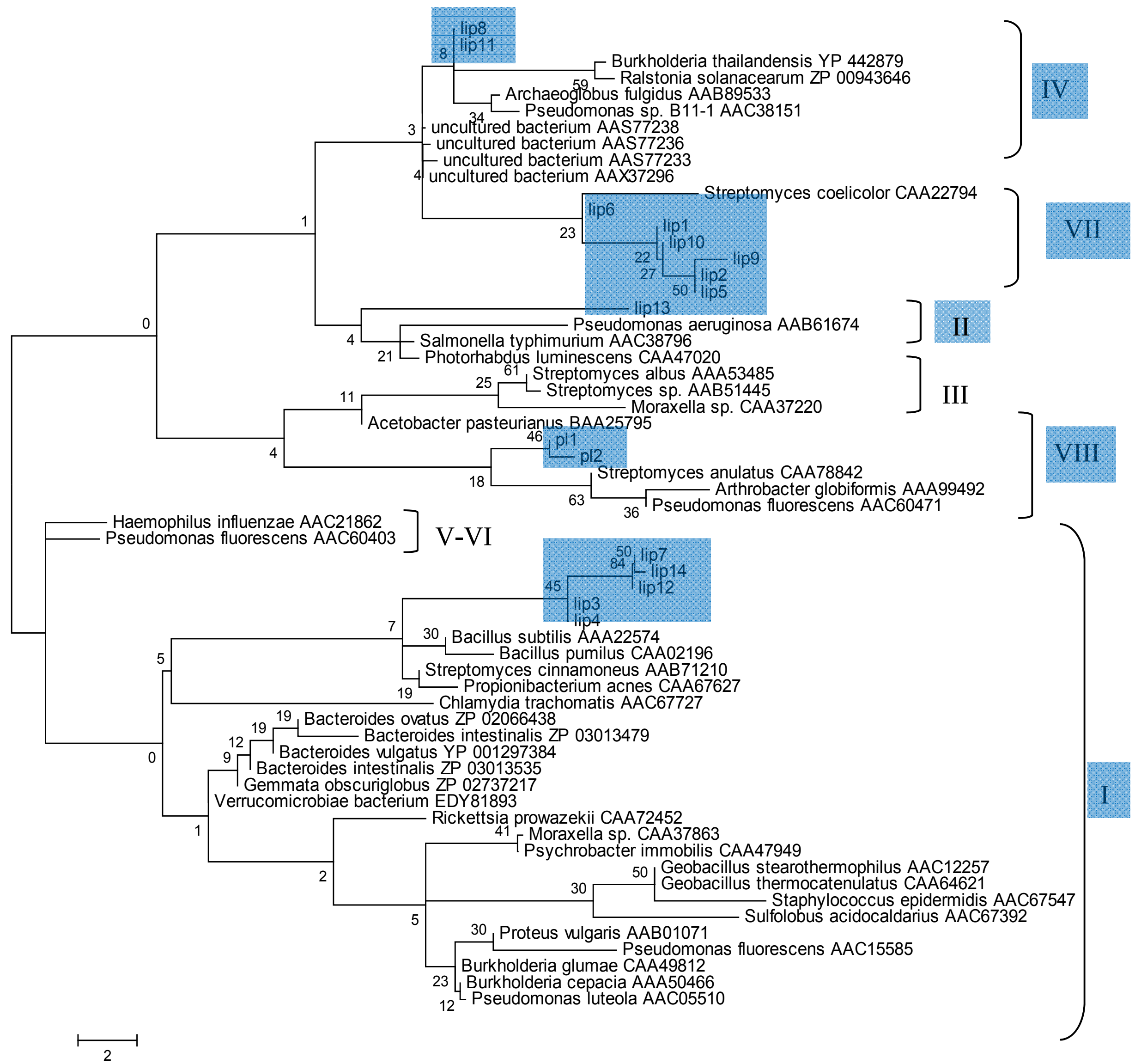
Discovery of lipases/esterases from rumen bacterial metagenomes



Positive clones sequenced =
16 putative lipases/esterases
(little homology to culturable sequenced rumen bacteria)

Privé F, Kaderbhai NN, Golyshina OV, Golyshin PN, Scollan ND, Newbold CJ, Huws SA. Novel lipases isolated from a bovine rumen metagenome. *Appl Microbiol Biotech* 99:5475-5485. 2015.

Phylogenetic positioning of isolated lipases relative to lipases within categorised families



Privé *et al*. (2015). *Appl Microbiol Biotech* 99:5475-5485.

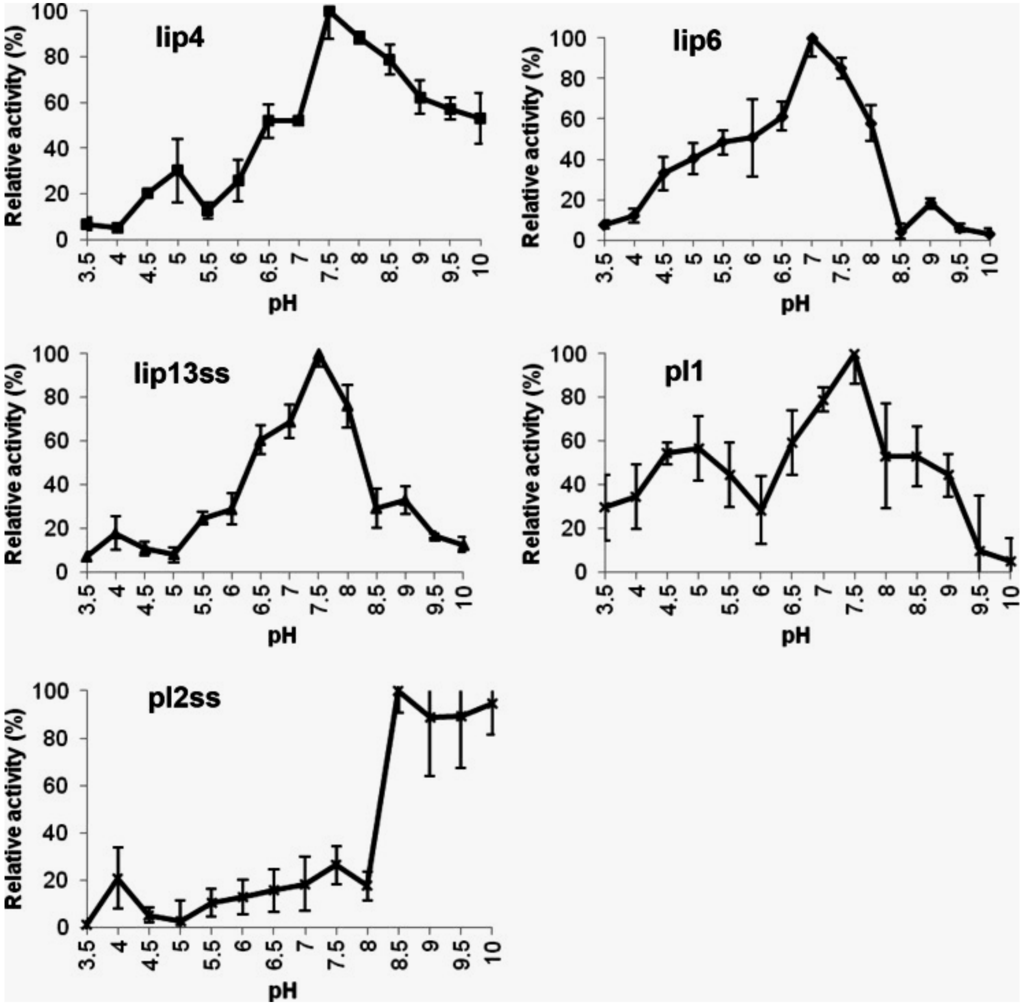
Lipase specificity of a selection of those discovered

Substrate	Specific activity (U/mg protein)				
	lip4	lip6	lip13ss	pl1	pl2ss
pNP-acyl esters					
Butyrate (C4)	56.3 ± 12.1	273.3 ± 22.5	ND	247.8 ± 11.1	172.5 ± 12.0
Caproate (C6)	28.7 ± 24.9	198.6 ± 10.8	51.1 ± 14.5	154.9 ± 21.8	58.8 ± 20.4
Caprylate (C8)	36.0 ± 23.7	42.4 ± 16.7	20.5 ± 14.4	317.5 ± 31.6	141.2 ± 17.0
Caprate (C10)	107.7 ± 37.3	30.5 ± 5.1	214.6 ± 14.5	224.6 ± 5.5	109.8 ± 4.5
Laurate (C12)	373.4 ± 45.7	23.8 ± 8.7	398.6 ± 7.1	224.6 ± 11.0	274.5 ± 36.3
Myristate (C14)	71.7 ± 12.4	18.7 ± 4.3	153.3 ± 25.6	209.1 ± 20.4	227.4 ± 27.2
Palmitate (C16)	ND	13.6 ± 7.0	ND	162.6 ± 32.4	235.3 ± 67.9
Stearate (C18)	ND	ND	ND	46.5 ± 32.8	109.8 ± 29.7

All showed some activity against the triglycerides Tributyrin (C4), Tricaprylin (C8) and Triolein (C18:1)

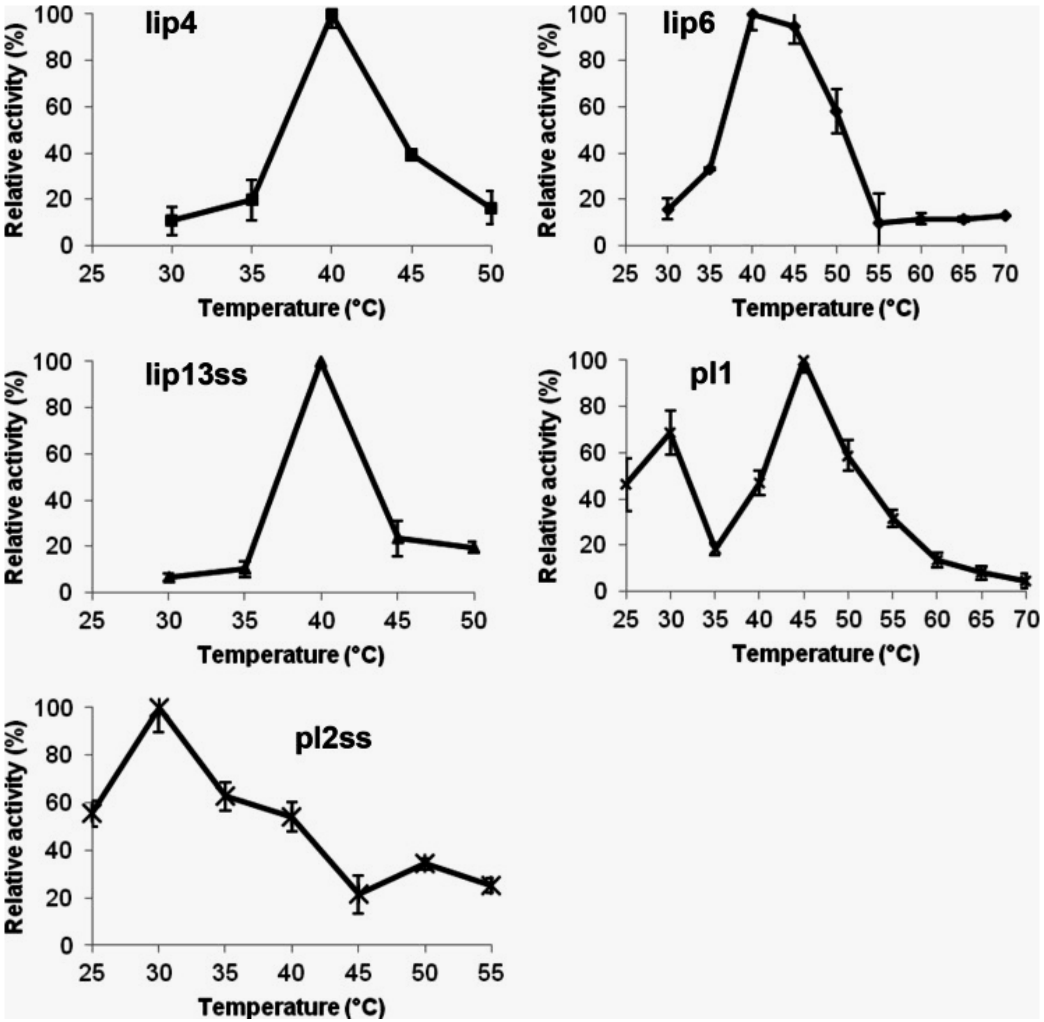
Privé *et al*. (2015). *Appl Microbiol Biotech* 99:5475-5485.

Isolated lipases varying degrees of pH tolerance



Privé *et al*. (2015). *Appl Microbiol Biotech* 99:5475-5485.

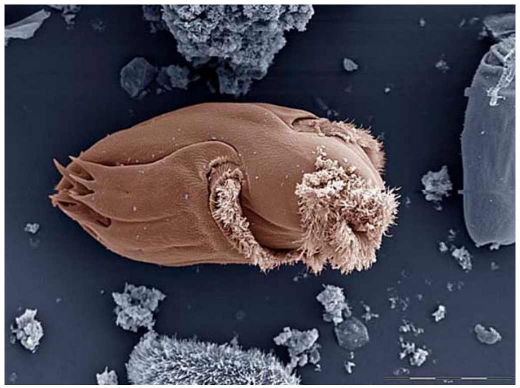
Isolated lipases varying degrees of temperature tolerance

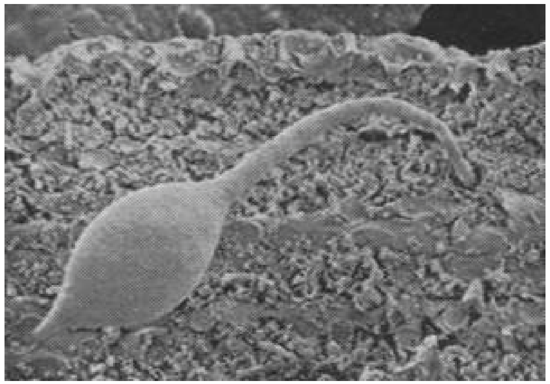


Privé *et al*. (2015). *Appl Microbiol Biotech* 99:5475-5485.

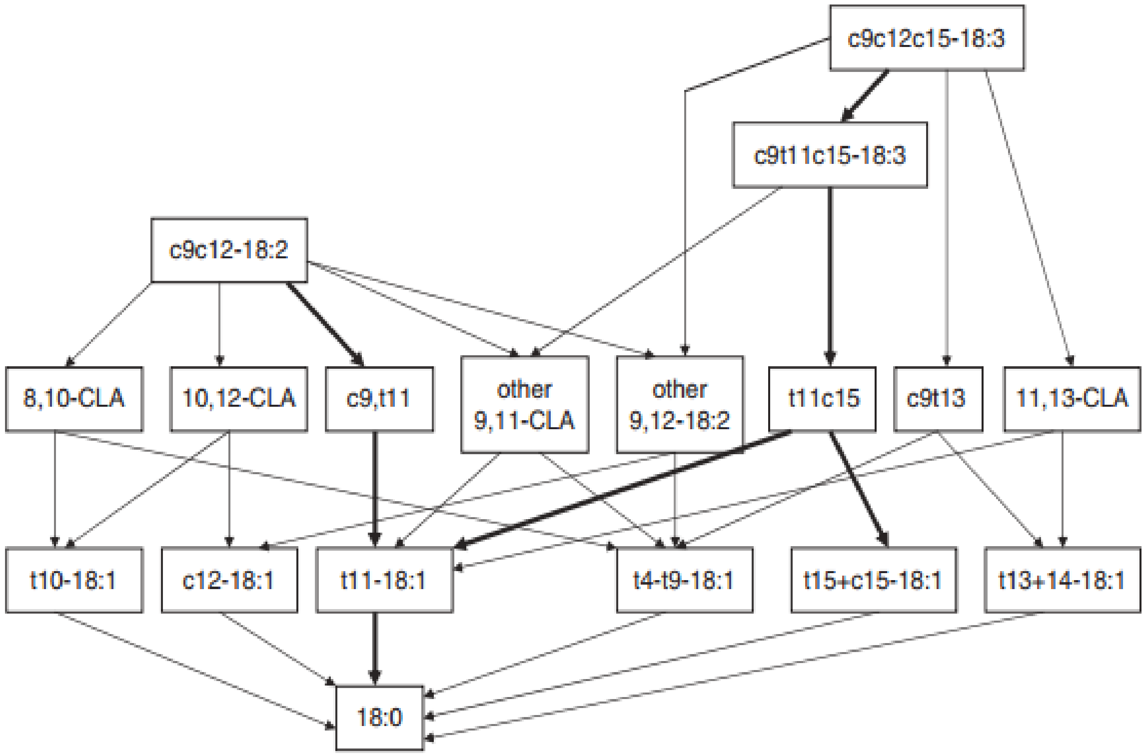
Contribution of the eukaryotome to rumen lipolysis

- No evidence that the rumen fungi play a role, but understudied.
- Rumen protozoal contribution disputed due to contamination of assays by bacteria.

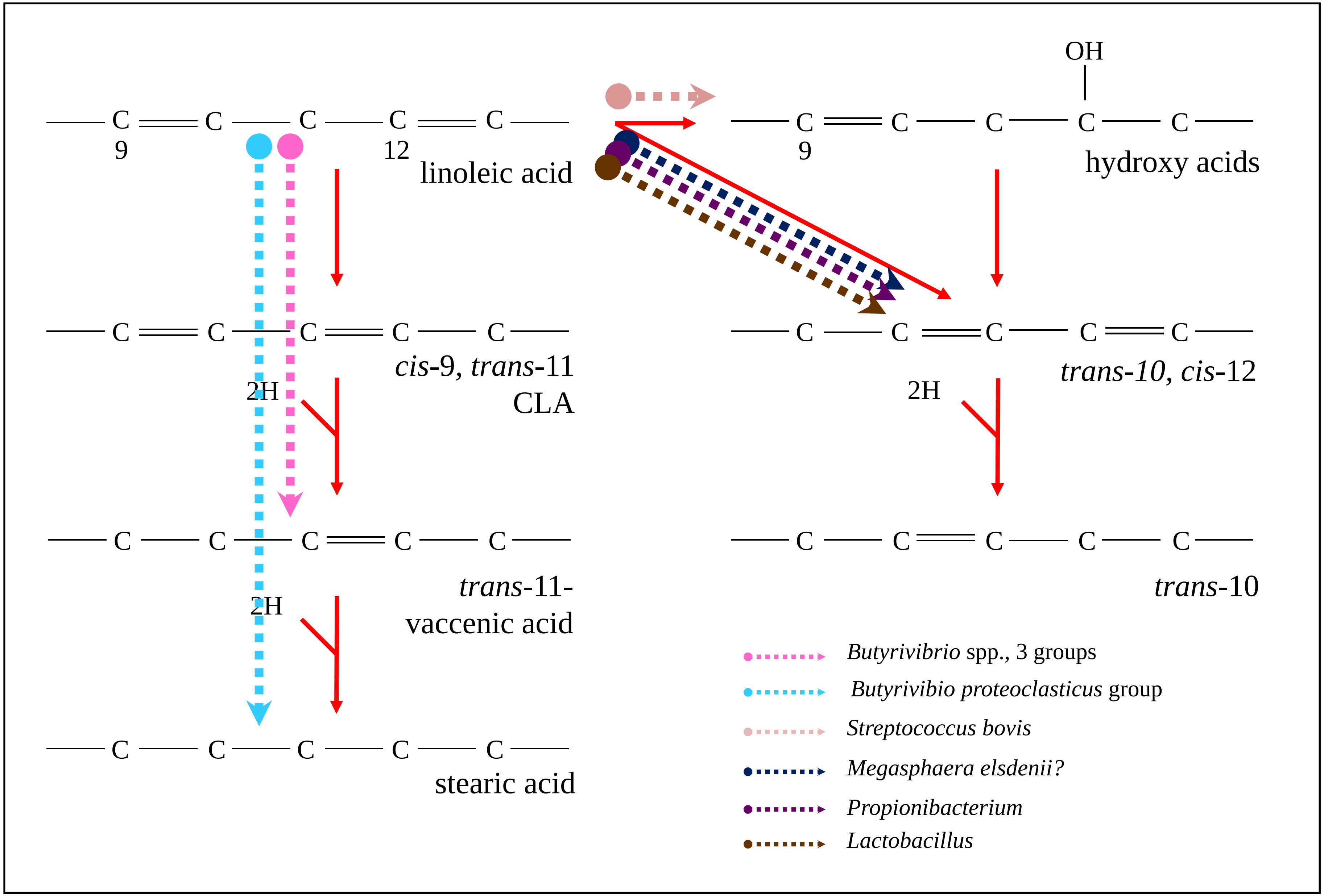




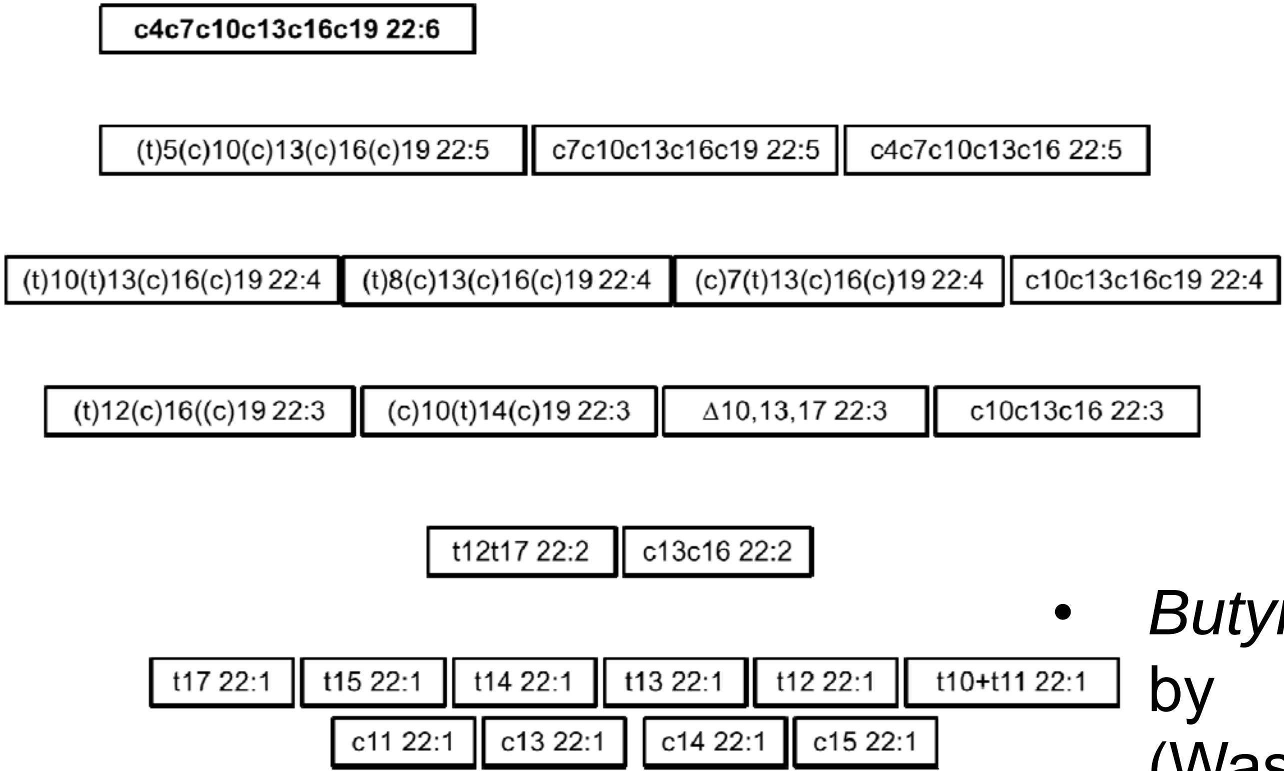
Understanding of rumen microbial biohydrogenation



Historical understanding of rumen bacterial biohydrogenation



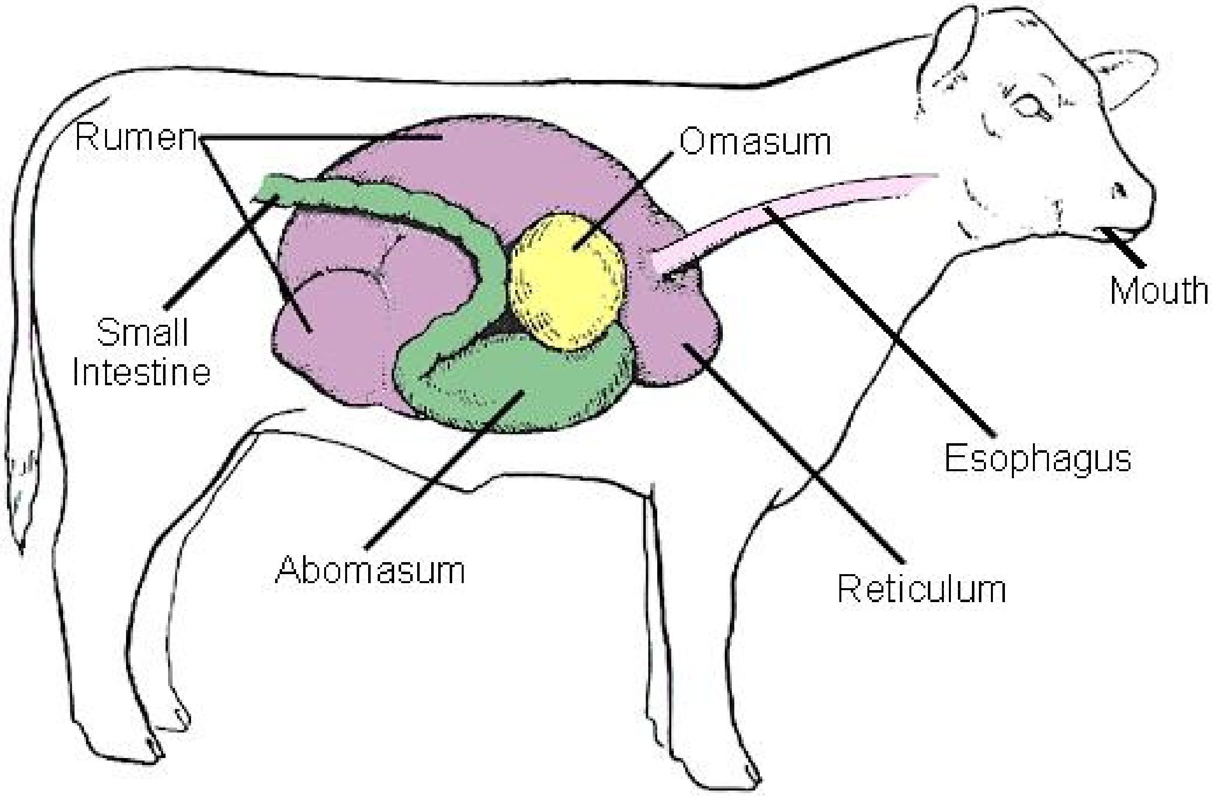
Biohydrogenation of long chain PUFA (LCPUFA)



- *Butyrivibrio* spp. inhibited by fish oil *in vitro* (Wasowska et al., 2006)

- At lower concentrations of DHA *Butyrivibrio* spp. again implicated *in vitro.* (Jeynathan et al., 2016)
- *Clostridium bifermentans* also able to convert LCPUFA (Sakurama et al., 2014)

Is the situation this simple *in vivo*?



Role of *Butyrivibrio* on biohydrogenation *in vivo*

Intervention	Animal type	Reduced biohydrogenation	Link to *Butyrivibrio*?	Reference
Ricinoleic acid	Sheep	Y	Y	Ramos Morales *et al.* 2015
Cumin seed extract	Goats	y	N	Miri *et al*., 2015
Sunflower oil and palm oil	Cows	y	N	Vargas-Bello Perez *et al.* 2016
Echium and linseed oil	Steers	y	N	Huws et al., 2015
Fish oil	Cows	y	y	Shingfield *et al*., 2012
Fish oil	Steers	Y	N	Kim *et al*., 2008
Fish oil	Steers	Y	N	Huws *et al*., 2011
Algae	Cows	Y	Y	Boeckaert *et al*., 2008
Algae	Goats	Y	N	Zhu *et al*. 2016

Our Fish oil study

3 × 3 repeated Latin Square with two animals per treatment.

Treatments:

F0 – Fish oil control (0% Dry Matter Intake (DMI))
F1 – Fish oil 1% DMI
F3 – Fish oil 3% DMI

Digesta flow at the duodenum was estimated using a dual-phase marker technique with ytterbium acetate and chromium ethylene diamine tetra acetic acid.

LABs and SABs collected from rumen fluid for microbial analysis on rRNA basis

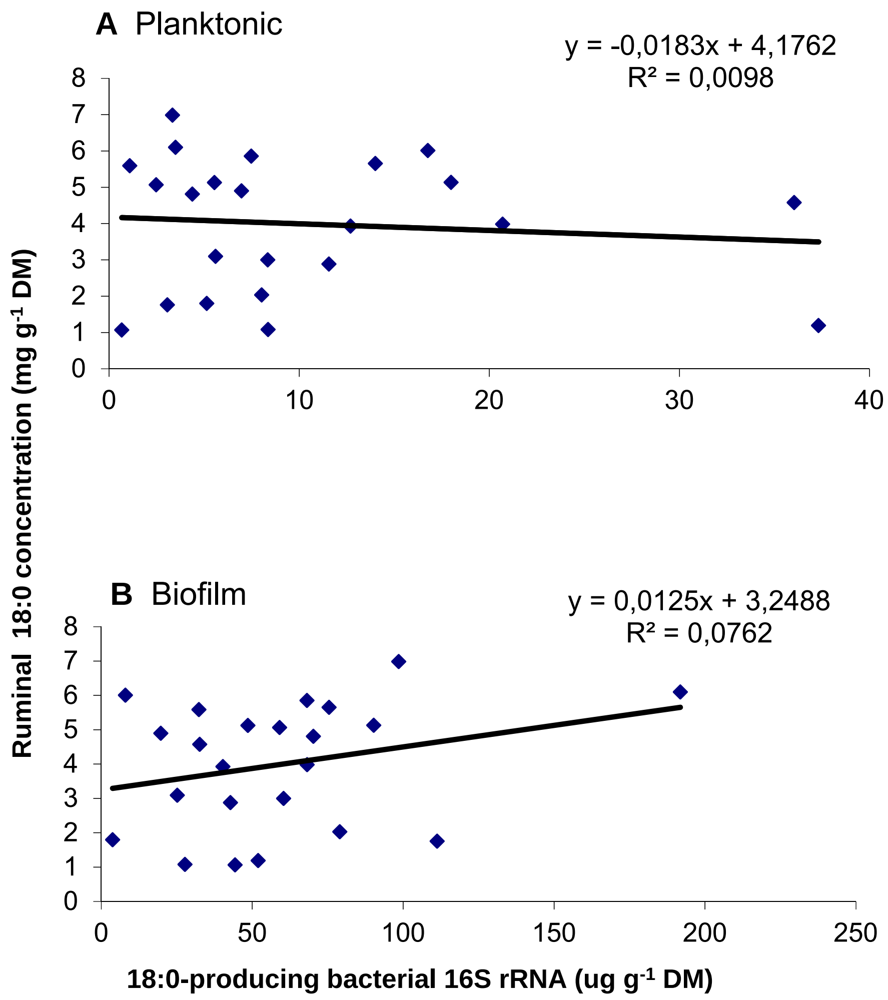
Huws et al. (2011). Environmental Microbiology

Concentration of key fatty acids within the rumen of steers receiving diets containing 0 (F0), 1 (F1) or 3% (F3) fish oil

	Diets				Significance
	F0	F1	F3	S.E.D	
Fatty acid content (mg g^{-1} DM)					
16:0	6.01	6.29	6.05	0.509	NS
18:0	5.25^{a}	4.47^{b}	2.07^{c}	0.638	<.001
18:1 *trans*-11	0.84^{a}	1.64^{b}	4.18^{c}	0.345	<.001
18:1 *trans*-10	0.11^{a}	0.16^{a}	0.26^{b}	0.028	<.001
18:2 *cis*-9, *trans*-11	0.03^{a}	0.04^{b}	0.05^{b}	0.006	0.003
18:2 *trans*-10, *cis*-12	0.01	trace	0.01	0.002	NS
18:2(n-6)	1.55^{a}	1.58^{a}	1.16^{b}	0.125	0.009
18:3(n-3)	1.20^{a}	1.31^{a}	0.81^{b}	0.117	0.002
Total LC (C20+)	0.34^{a}	0.81^{b}	1.50^{c}	0.092	<.001
Total fatty acids	20.2	22.6	23.9	1.90	NS

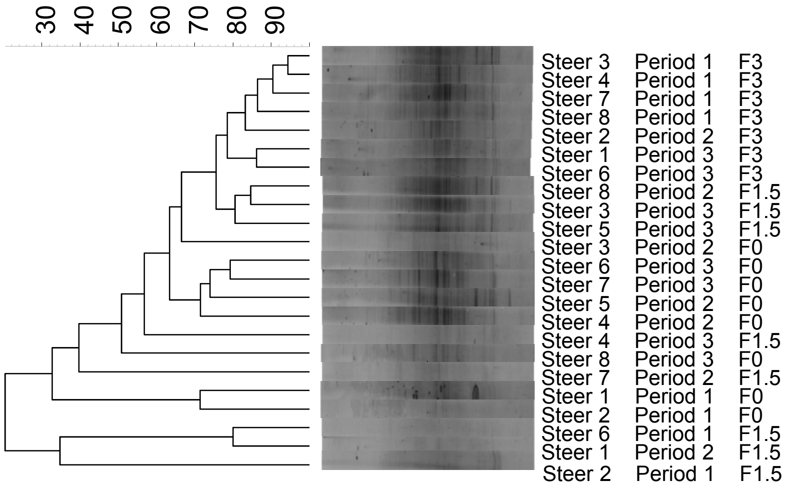
NS – Not Significant

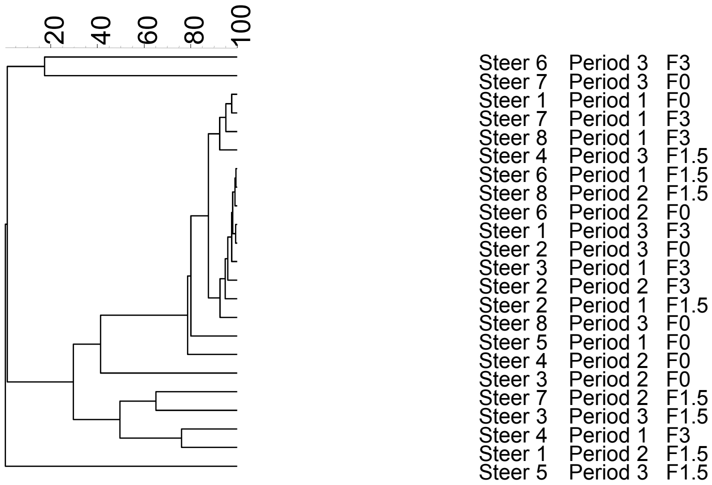
Values with different superscripts are significantly different (*P*>0.05).

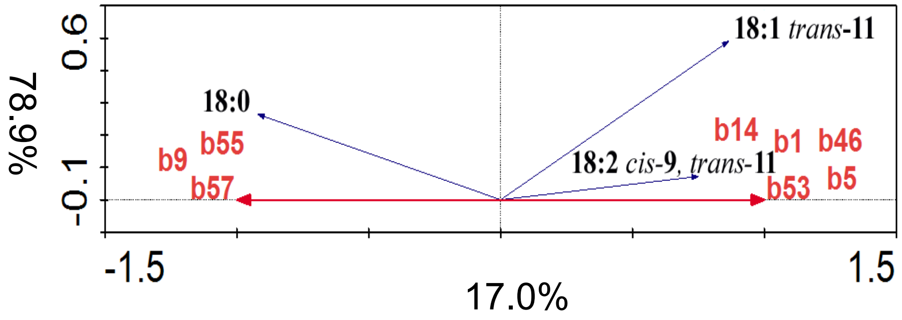


Quantitative PCR data showing correlation between *C. proteoclasticum* 16S rRNA gene concentration and ruminal18:0 concentration for planktonic (LAB; A) and biofilm (SAB;B) samples.

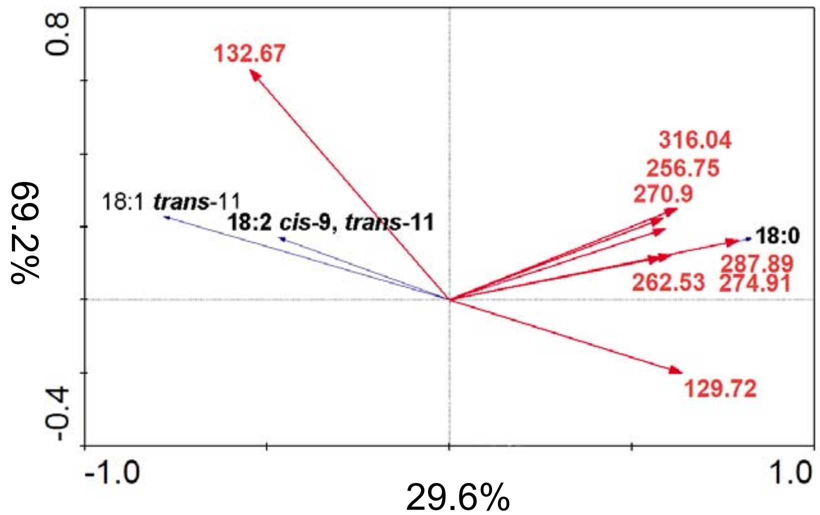
As yet uncultured *Prevotella*, *Lachnospiraceae* incertae sedis, and unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* may play a predominant role in ruminal biohydrogenation







F0 - 0% fish oil
F1.5 – 1.5% Fish oil
F3 – 3% Fish oil



Huws *et al*., 2011. *Environmental Microbiology*, 13:1500-12

Echium oil

- Conversion of 18:3n-3 to 18:4n-3 is important (rate-limiting) for EPA and DHA synthesis in liver

- 18:4n-3 is rich in Echium oil

18:3n-3
Alpha-linolenic acid

↓

18:4n-3

↓

20:4n-3

↓

20:5n-3

↓

22:5n-3
Eicosapentaenoic acid
(EPA)

↓

22:6n-3
Docosahexaenoic acid
(DHA)

Echium oil and flaxseed oil study

3 × 3 repeated Latin Square with two animals per treatment.

Treatments:

GS – Grass silage
GSE – 3% DMI Echium oil and grass silage
GSF – 3% DMI flaxseed oil and grass silage

Rumen fatty acids sampled for fatty acid and microbial analysis 2 h after feeding on day 21 of the period.

Huws et al. (2015). Microbial Biotechnology

Rumen fatty acid profiles in steers fed grass and sugar beet (GS), and GS with the addition of flax (GSF) or echium oil (GSE)[1]

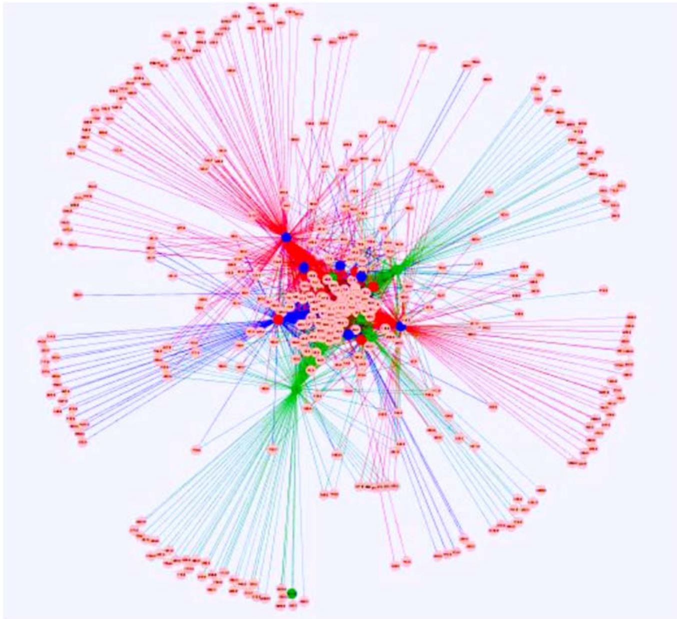
	Diets				
Fatty acid	GS	GSF	GSE	SED	*P*
Branched and Odd chain fatty acids (BOC)	1.437[a]	1.450[a]	1.547[a]	0.060	0.231
12:0	0.612[a]	0.651[a]	0.653[a]	0.03	0.350
14:0	0.312[a]	0.336[b]	0.334[b]	0.007	0.016
16:0	3.207[a]	4.251[b]	4.980[c]	0.140	<0.001
18:0	4.193[a]	9.783[b]	11.061[b]	0.580	<0.001
18:1 *trans*-6,-7,-8	0.024[a]	0.246[b]	0.253[b]	0.009	<0.001
18:1 *trans*-9	0.019[a]	0.166[b]	0.217[c]	0.006	<0.001
18:1 *trans*-10	0.030[a]	0.201[b]	0.246[c]	0.001	<0.001
18:1 *trans*-11	0.555[a]	2.999[b]	4.797[c]	0.320	<0.001
18:1 *trans*-12	0.034[a]	0.231[b]	0.291[c]	0.013	<0.001
Sum 18:1 trans	0.850[a]	4.926[b]	6.832[c]	0.352	<0.001
18:1 *cis*-9	0.606[a]	3.423[b]	3.419[b]	0.176	<0.001
18:1 *cis*-11	0.089[a]	0.120[b]	0.221[c]	0.008	<0.001
18:1 *cis*-12	0.010[a]	0.082[b]	0.051[c]	0.008	<0.001
18:1 *cis*-13	0.011[a]	0.027[b]	0.030[b]	0.003	<0.001
Sum 18:1 cis	0.126[a]	0.378[b]	0.388[b]	0.021	<0.001
18:2 *cis*-9, *trans*-11	0.029[a]	0.274[b]	0.332[c]	0.022	<0.001
18:2 *trans*-9, *trans*-12	0.009[a]	0.054[b]	0.029[c]	0.006	<0.001
18:2 *cis*-9, *cis*-12	1.429[a]	2.162[b]	2.331[b]	0.238	0.015
18:2 *trans*-10, *cis*-12	0.018[a]	0.019[a]	0.021[a]	0.003	0.538
18:2 *trans*-11, *trans*-13	0.016[a]	0.124[b]	0.125[b]	0.011	<0.001
Sum 18:2 Conjugated linoleic acid	0.081[a]	0.470[b]	0.579[c]	0.033	<0.001
18:2*n*-6	1.429[a]	2.162[b]	2.331[b]	0.240	0.020
18:3*n*-3	1.104[a]	3.670[b]	2.970[b]	0.374	<0.001
18:4*n*-3	0.049[a]	0.041[a]	1.261[b]	0.170	<0.001
20:0	0.190[a]	0.248[b]	0.257[b]	0.011	<0.001
20:4	ND	ND	ND	NA	NA
20:5	0.000[a]	0.008[b]	0.013[c]	0.000	<0.001
22:5	ND	ND	ND	NA	NA
22:6	ND	ND	ND	NA	NA
Sum LCPUFA (C20 and above)	0.681[a]	0.921[b]	1.166[c]	0.082	0.001
Total fatty acids	13.72[a]	35.75[b]	38.97[b]	2.145	<0.001

Roseburia and Succinivibrio may be involved in the biohydrogenation of 18:4n-3

Sequencing information
- 724,785 reads pre-QIIMME
- 570, 483 reads post-QIMME
- 95,468 average reads/sample for GS diet
- 187,368 average reads/sample for GSE diet
- Average read length 377bp
- Average OTUs per sample for GS rumen samples 5,095
- Average OTUs per sample for GSE rumen samples 5,972

	Diet			
Genus	GS	GSE	SED	P
Streptomyces	0.024[a]	0.014[b]	0.007	0.052
Bacteroidales; other	0.031[a]	0.0007[b]	0.005	0.007
Bacteroidetes; other	0.061[a]	0.027[b]	0.012	0.010
Prevotella	0.102[b]	0.058[ab]	0.020	0.027
Anaerolinea	0.020[a]	0.011[b]	0.00	0.028
Roseburia	0.007[a]	0.024[b]	0.005	0.001
Eubacteriaceae;Other	0.034[b]	0.016[a]	0.007	0.046
Victivallis	0.040[a]	0.019[b]	0.007	0.016
Succinivibrio	0.001[a]	0.010[b]	0.002	0.014
Proteobacteria; Other	0.111[a]	0.063[b]	0.013	0.015

Only genera showing significant differences are shown in the table ($P<0.05$) (Data shown are % occurrences within the total reads). GS – Grass silage diet; GSE - Grass silage diet with echium oil supplementation.



On an OTU level animal variation was the main cause of difference in microbiome seen

Huws *et al. (2015)* Microbial Biotechnology

Biohydrogenation and rumen microbiome involvement differ dependent on animal type?

- Recent publication from Toral et al. (2016) suggests that the animal genotype plays a role in biohydrogenation capacity due to differences in the rumen microbiome.

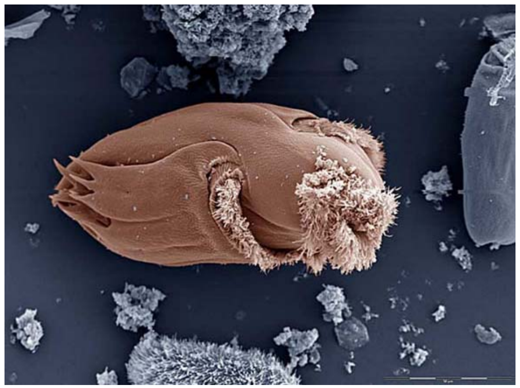
- Data shows that the cow rumen lipidome was more concentrated in 18:1 trans-10 than that of goats following feeding with 5.3% sunflower oil and additional starch. More prone to milk fat depression.

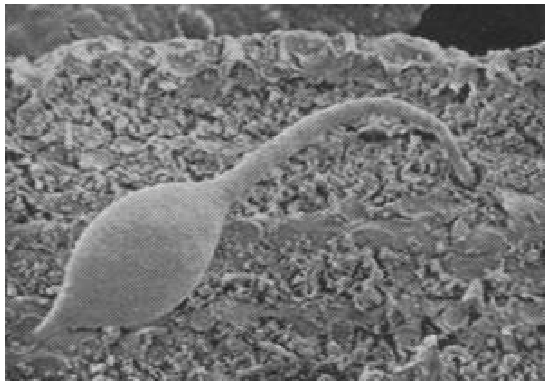
- In caprine lipidome 2.2% fish oil dietary supplementation had more of an effect in terms of 18:1 trans-11 formation and decreases in 18:0 than that seen in bovine rumen lipidome.

Role of the rumen eukaryotome in rumen biohydrogenation

- Evidence suggests rumen protozoa do not play a role (Devilliard et al., 2006)

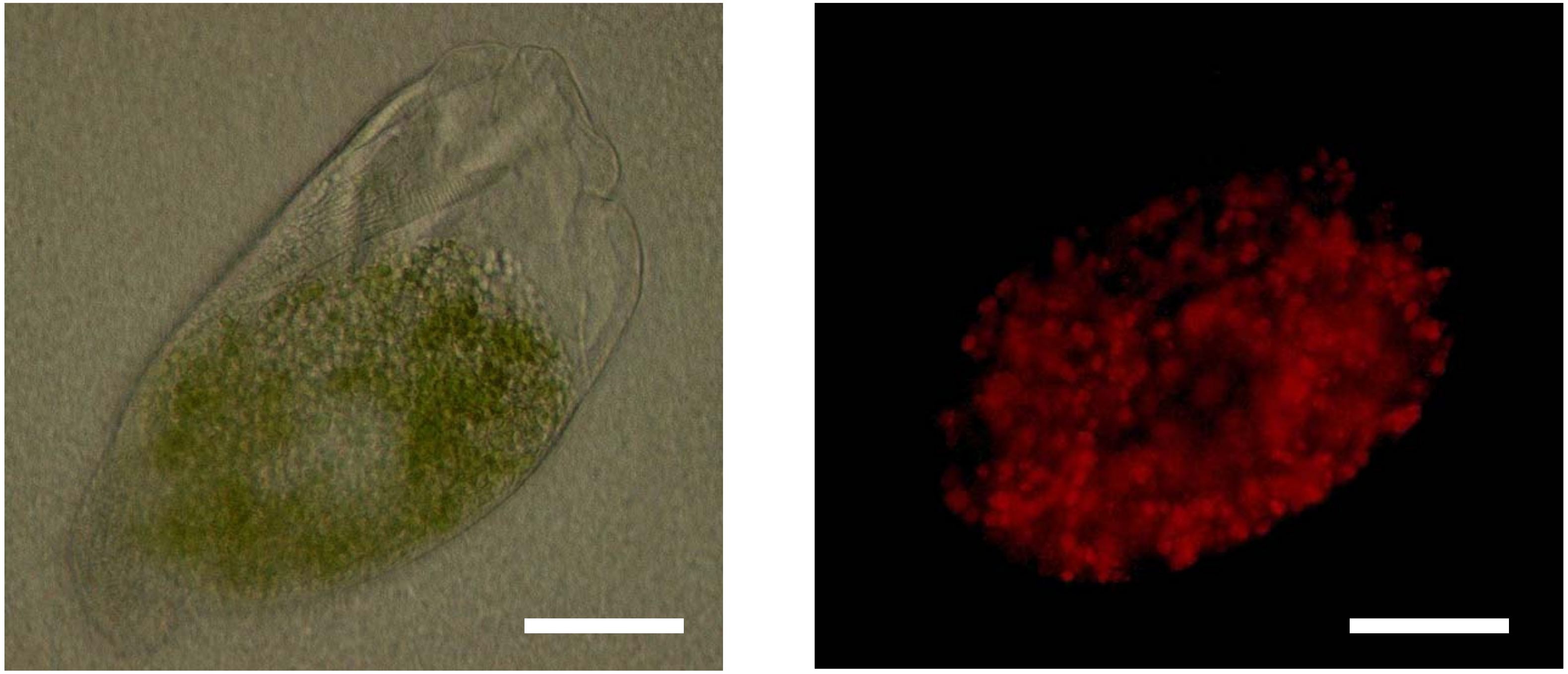
- Data published by Nam and Garnworthy (2007) that rumen protozoa, particularly Orpinomyces, have the capacity to biohydrogenate, albeit slowly.





Interactions of the rumen protozoa with the plant chloroplast

Ruminal protozoa are rich in polyunsaturated fatty acids (PUFA) due to ingestion of PUFA-rich chloroplast



***Epidinium* sp. isolated from the rumen of steers fed fresh grass**

Scale bars – 20 μm

Huws *et al.* (2009). *FEMS Microbiology Ecology*, 69: 461-471.

Protozoal chloroplast and 18:3 n-3 content post feeding of steers on high and low chloroplast content forages

	Diet S/C (straw/concentrate)	Diet PRG (Fresh Perennial Ryegrass)
Period 1	Steer 1, 2, 3	Steer 4, 5, 6
Period 2	Steer 4, 5, 6	Steer 1, 2, 3

Low chloroplast content

High chloroplast content

	Diet: Straw/Conc	Diet: Grass	SED	*P*-value
Protozoal density (10^3 cells mL^{-1})				
Total	256	367	0.295[2]	NS
Holotrich	ND	ND	NA	NA
Entodiniomorphid	256	367	0.295[2]	NS
% protozoa containing intracellular chloroplasts				
Total	22.5	31.8	1.39	*
Holotrich	ND	ND	ND	NA
Entodiniomorphid	22.5	31.8	1.39	*
% protozoa saturated with intracellular chloroplasts (>10 $cell^{-1}$)				
Total	2.3	5.0	1.17	NS
Holotrich	ND	ND	NA	NA
Entodiniomorphid	2.3	5.0	1.17	NS
Protozoal standard				
Protozoal chlorophyll content (µg mL^{-1})	1.0	1.1	0.68	NS
Protozoal N content (mg/g)	0.48	0.33	0.16	**
DNA:N	5.22	3.47	2.19	NS
Protozoal fatty acid content (ug/mg N)				
14:0	0.68	0.90	0.11	*
15:0	0.40	0.54	0.20	*
16:0	16.6	8.11	1.96	*
17:0	0.34	0.25	0.13	*
18:0	22.7	12.6	15.0	NS
Trans-11 18:1	3.69	4.26	2.61	*
18:2 n-6	tr	tr	NA	NA
18:3 n-3	0.36	1.41	0.36	*
Cis-9, *trans*-11 CLA	0.43	0.05	0.18	*
Trans-10, *cis*-12 CLA	tr.	tr.	NA	NA
Total Fatty acids	65.1	38.7	21.81	NS

Protozoal contribution to fatty acid flow following feeding of steers steers on high and low chloroplast content forages

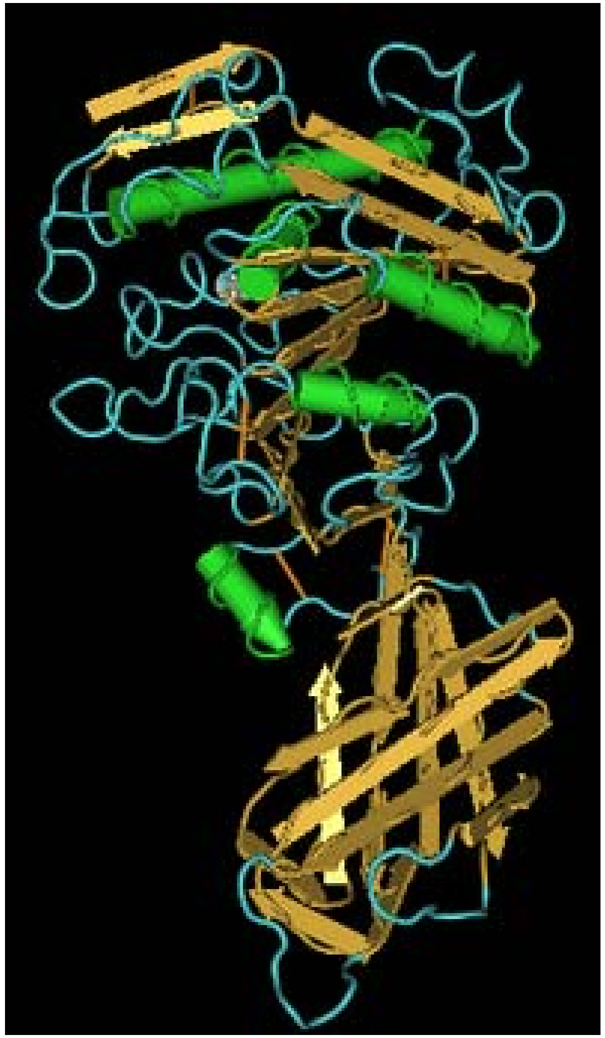
	Duodenal flow (g/d)				Protozoal flow (g/d)				Contribution*	
	S/C	PRG	SED	*P*	S/C	PRG	SED	*P*	S/C	PRG
Fatty acids										
14:0	2.29	1.78	0.40	**	0.22	0.00	0.00	*	22.2	0.00
15:0	1.44	1.38	0.19	*	0.13	0.00	0.04	*	9.02	0.00
16:0	24.9	27.1	3.13	*	5.44	0.03	0.46	*	21.8	0.00
17:0	1.32	1.62	0.30	*	0.11	0.00	0.02	*	8.33	0.00
18:0	88.6	108	13.9	*	7.30	0.10	3.33	NS	8.23	0.00
Trans-11 18:1	5.20	25.1	4.16	*	1.20	0.02	0.18	*	23.1	0.00
18:2 n-6	10.2	2.25	0.68	*	1.16	0.00	0.15	*	11.4	0.00
18:3 n-3	1.66	3.12	0.46	*	0.12	0.00	0.04	*	7.23	0.00
Cis-9, *trans*-11 CLA	0.10	0.08	0.04	*	0.08	0.00	0.05	*	80.0	0.00
Trans-10, *cis*-12	ND	0.07	0.02	*	0.00	0.00	0.00	*	0.00	0.00
Total Fatty acids	173	207	20.7	*	21.1	0.10	3.58	*	0.01	0.00

Retention of protozoa within the rumen following grass feeding

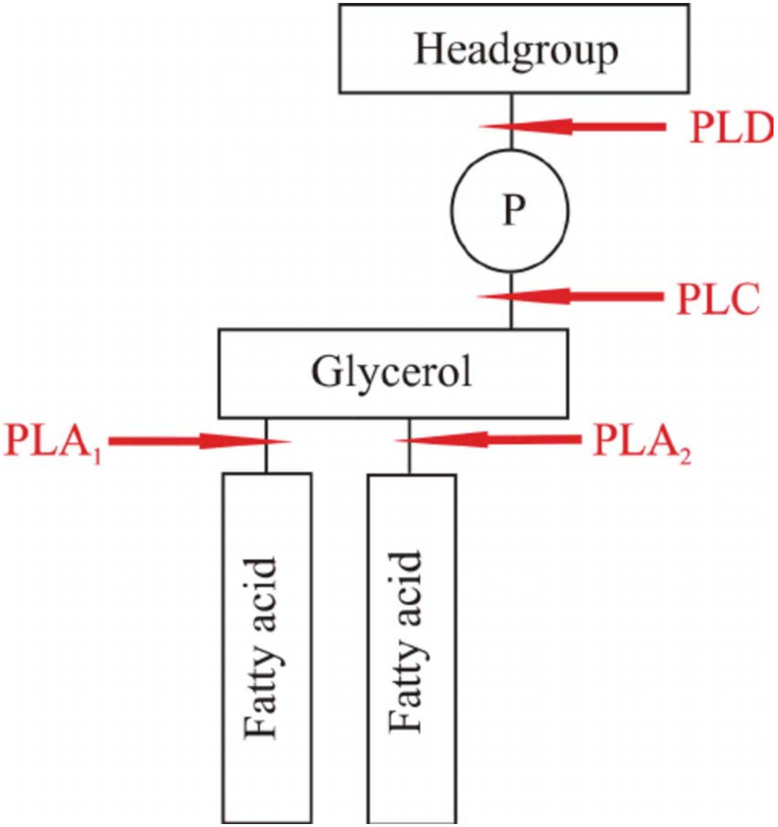
Challenge - enhance chloroplast uptake whilst ensuring duodenal flow

Possible future directions?

- LIPASES - Novel targets for inhibition of biohydrogenation:
- Inhibit – More PUFA flow to duodenum?
- Enhance – More PUFA flow to duodenum?



Proof of principle: Effects of addition of PLA_1 on rumen lipid metabolism



75, 100, 125 and 150 µM phospholipase A1 added to *in vitro* incubations containing rumen fluid and phospholipids extracted from perennial ryegrass.

Effects on polar lipids

Polar fraction (phospholipids):

75, 100, 125 and 150μM phosphlipase A1:
↓ 18:3*n*-3 and 18:2*n*-6.
Likely fatty acids on position A1 of perennial ryegrass

Free non-esterified fatty acids:

Biohydrogenation 18:2*n*-6:
75μM: 75.8%
100μM: 85.0%
125μM: 80.4%
150μM :73.0%

Biohydrogenation 18:3*n*-3:
75μM: 74.6%
100μM: 87.5%
125μM: 15.2%
150μM :20.1%

Conclusions

- Rumen lipid metabolism is clearly very complicated, particularly biohydrogenation

- As a consequence, manipulation of biohydrogenating bacteria to modify the ‘healthiness’ of meat and milk is challenging at present.

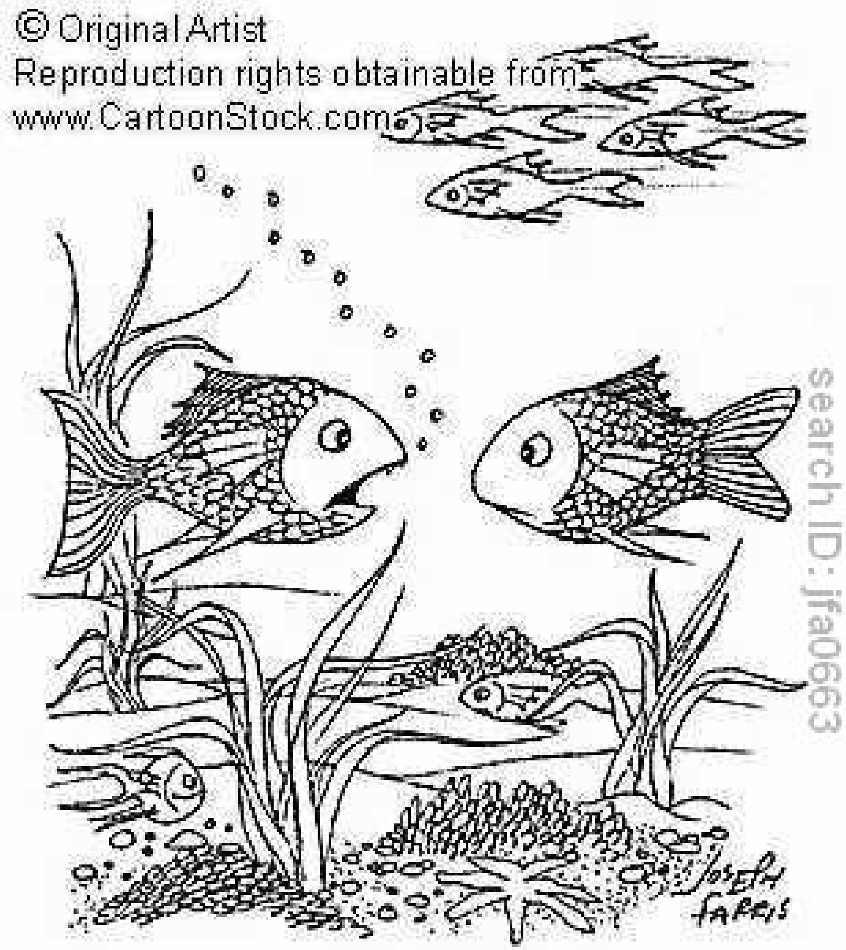
- Hungate 1000 genomes may help us understand the isomerases and reductases involved.

- Predict biohydrogenation capacity from microbiome diversity?

Conclusions

- Lipolysis could be an easier target?
- More work needs to be undertaken to understand rumen lipolysis
- Could we develop technologies to enhance protozoal bypass, whilst maintaining rumen numbers, in order to ensure more chloroplast flow to the duodenum?
- Novel technologies must consider effects on methane and production

Questions?



"Bad news! More people are switching from red meat to fish!"