Milk Metabolomics of Heat-Stressed Goats with and without Intramammary Lipopolysaccharide Challenge

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Responses of goats to heat stress (HS)

- **Thermophysiological traits:**
  - Rectal Temperature (+0.58ºC)
  - Respiratory Rate (+300%)
  - Water consumption (+202%)
  - Dry matter intake (-21%)
  - Body weight (-125 g/d)

- **Milk yield and milk quality:**
  - Milk yield (-4 to -10%)
  - Milk quality (–8 to12% fat & protein)
  - Cheese coagulation properties (> RCT)

Hamzaoui *et al*. (2012, 2013); Abdel-Gawad *et al*., 2014; Castro-Costa *et al*., 2015; Contreras-Jodar *et al*., 2018)

Transcriptomics of goat milk cells

- RNA-seq revealed **699** differentially-expressed genes by HS.

Component	Gen_id
Fat	ACACA
	FASN
	SCD
Protein & lactose	CSN1S1
	CSN1S2
	CSN3
	LTF
	LALBA
Cathepsins	CTSB
	CTSD
	CTSZ
	CTSS
	CTSL1
Plasminog.	PLAU
	PLAUR

Reduced de novo FA synthesis

Reduced casein synthesis

More susceptibility to mastitis?

Protein degradation
impaired milk coagulation

Salama et al. (2015)

Objectives

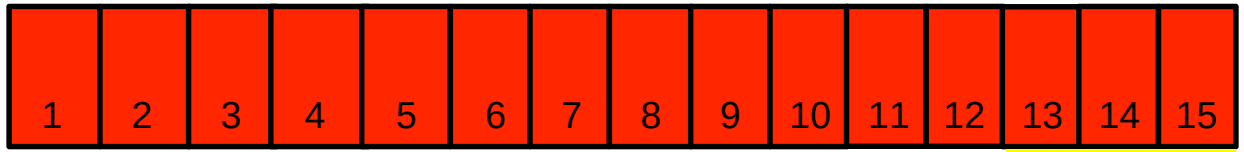
1. Whether heat stress would affect the immune response of the mammary gland to infection.
2. The use of ^{1}H NMR-based milk metabolomics to identify inflammation markers under:
   - Thermal-neutral (TN) conditions
   - Heat stress (HS) conditions

Experimental design

- **8 Murciano-Granadina dairy goats (42 ± 2 kg BW; 100 ± 5 DIM; 2.2 ± 0.1 L/d)**
- **Climatic chamber:**
  - **TN: 15 to 20°C, 45 to 55% HR; THI = 59 to 65**
  - **HS: 35°C-12h and 28°C-12h, 40% HR; THI = 83 (day) and 75 (night)**
- **Challenge with LPS :**
  - LPS half:**10 µg** in 2 ml 0.09% sterile saline
  - CON half: 2 ml 0.09% saline

Experimental design

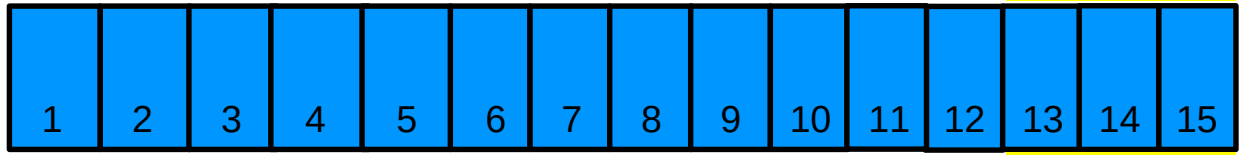
HS



HS-CON
HS-LPS

Saline
LPS

TN



TN-CON
TN-LPS

Saline
LPS

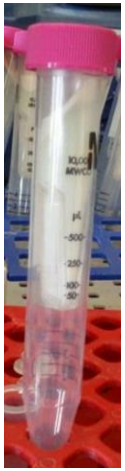
n = 8
4 goats / trt

d 1 to d 15:
– Milk yield
– Milk composition
– Feed and water intakes
– RT and RR

d 12 to 15:
– Milk: 0, 2, 4, 6, 8, 12, 24, 48, 72 h for yield and composition
– Milk: 0, 4, 6,12, 24 h for NMR metabolomics analysis

Milk sample preparation and NMR analysis (n=80)

Sample Preparation (Beckonert *et al.*, 2007)	
1. Buffer	Na_2HPO_4, NaH_2PO_4, NaN_3, D_2O
2. Milk Filtration	4 mL in 10 kDa filtration tube
3. Tube centrifugation	12,000 × g, 20 min at 22ºC
4. Sample	400 μΛ
5. Buffer	200 μΛ
6. Milk + Buffer mix	550 μΛ ιν 5 mm NMR tubes



Milk sample preparation and NMR analysis (n=80)

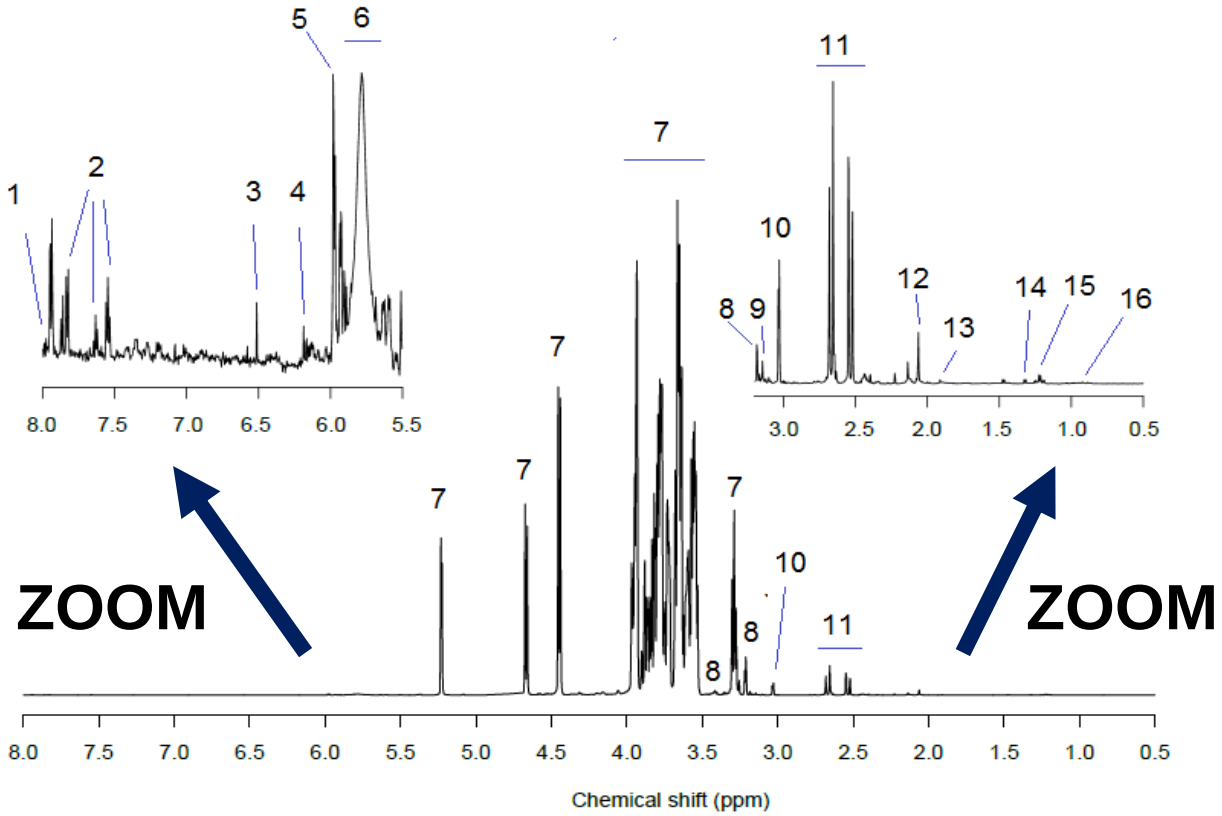
Sample Preparation (Beckonert *et al.*, 2007)	
1. Buffer	Na_2HPO_4, NaH_2PO_4, NaN_3, D_2O
2. Milk Filtration	4 mL in **10 kDa** filtration tube
3. Tube centrifugation	12,000 × g, 20 min at 22ºC
4. Sample	400 μΛ
5. Buffer	200 μΛ
6. Milk + Buffer mix	550 μΛ ιν 5 mm NMR tubes
^{1}H nuclear magnetic resonance (NMR) analysis	
1. Bruker Avance-III spectrometer (Bruker BioSpin, DE)	NMR **600 MHz** ^{1}H; 298ºK NOESY pulse sequence
2. Bruker TopSpin software v2.1.	Data acquisition Data processing

Bioinformatic and statistical analysis

1.	**Bioinformatics**	• Baseline correction
		• Region of interest: δ 8.0 – 5.0 and 4.6 – 0.5 ppm
		• Binning: interval width from 0.0003 to 0.0300 ppm
		• Normalization: mean centered, logarithm transf.
2.	**Statistics**	Multivariate analysis : PCA and PLS-DA
		Volcano Plot
3.	**Metabolite Id**	Previous bibliography (Sundekilde *et al.*, 2013) Human Metabolome Database (Wishart *et al.*, 2009)

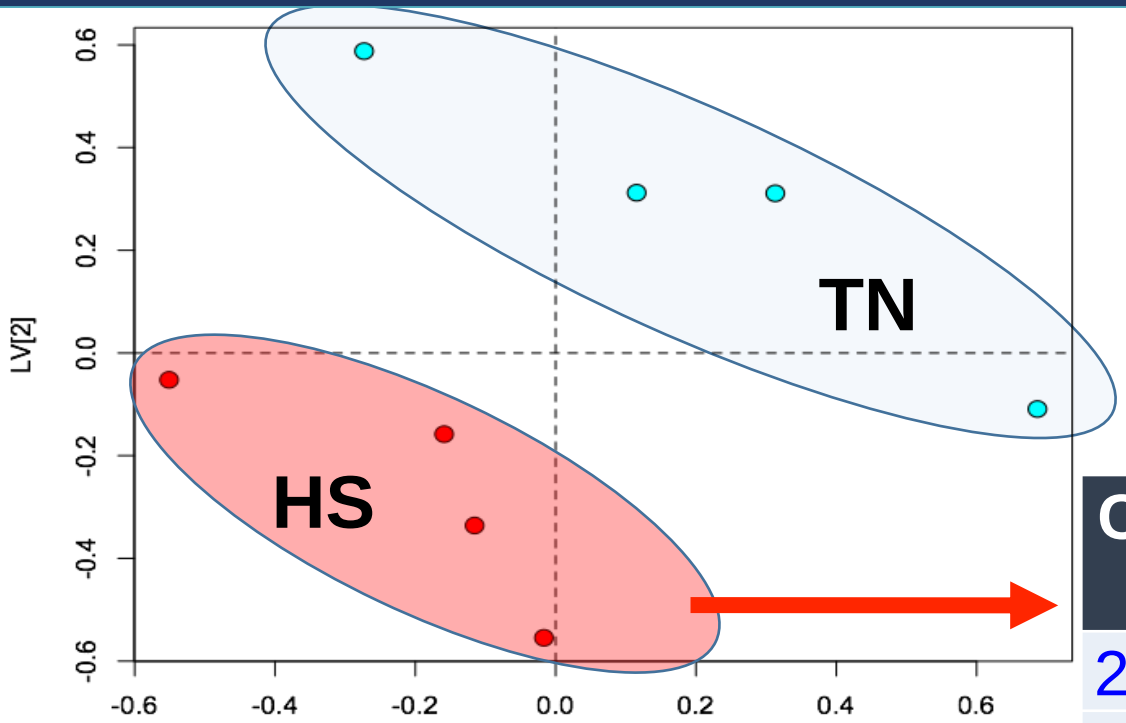
R software 3.2.3 version
(R Core Team, 2015)

600 MHz ^{1}H NMR Metabolomics in Milk



1. 3-metylhistidine
2. Hippurate
3. Fumarate
4. Orotate
5. Maleate
6. Urea
7. Lactose
8. Carnitine
9. Creatinine
10. Choline
11. Citrate
12. N-acetylcarbohydrates
13. Acetate
14. Lactate
15. b-hydroxybutyrate
16. Butyrate

PLSDA Scores: TN-CON vs HS-CON (markers of HS)

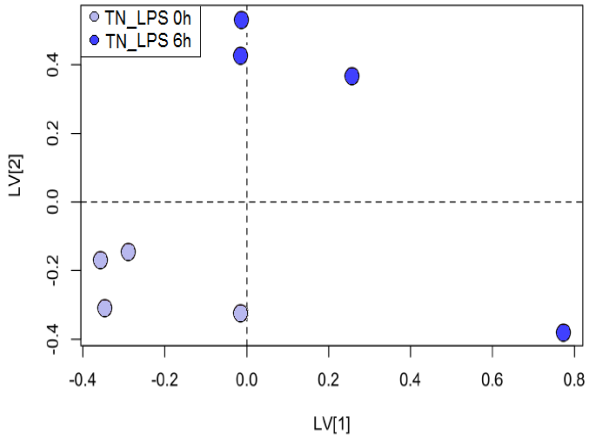


$R_x^2 = 0.36$
$R_y^2 = 0.60$
$R^2_{cum} = 0.96$
$Q^2 = 0.24$

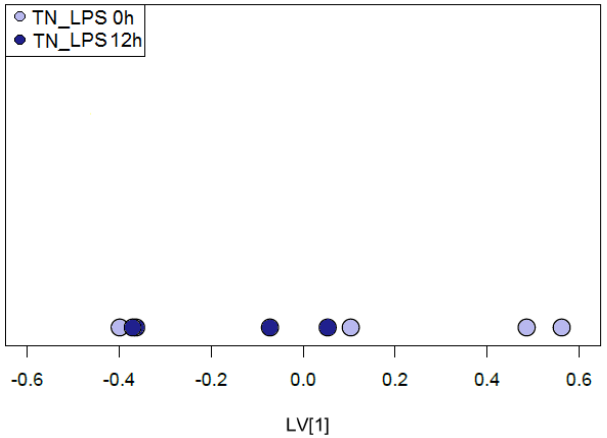
Chemical shift (ppm)	Metabolite
2.52, 2.55, 2.68	Citrate
4.37	Unknown
3.35	Unknown

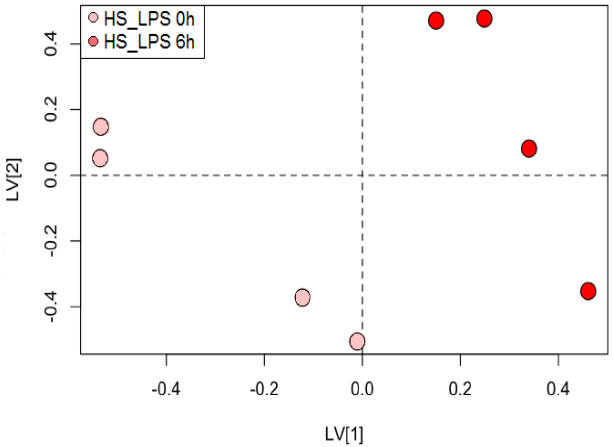
Negative relationship with milk coagulation

PLSDA plot: response to LPS throughout time

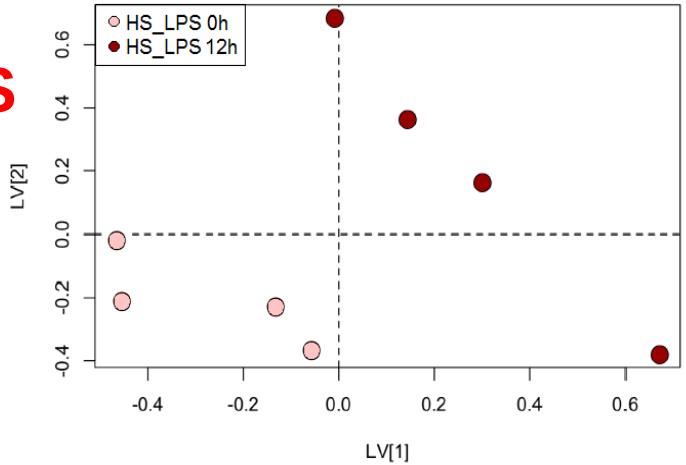


TN





HS

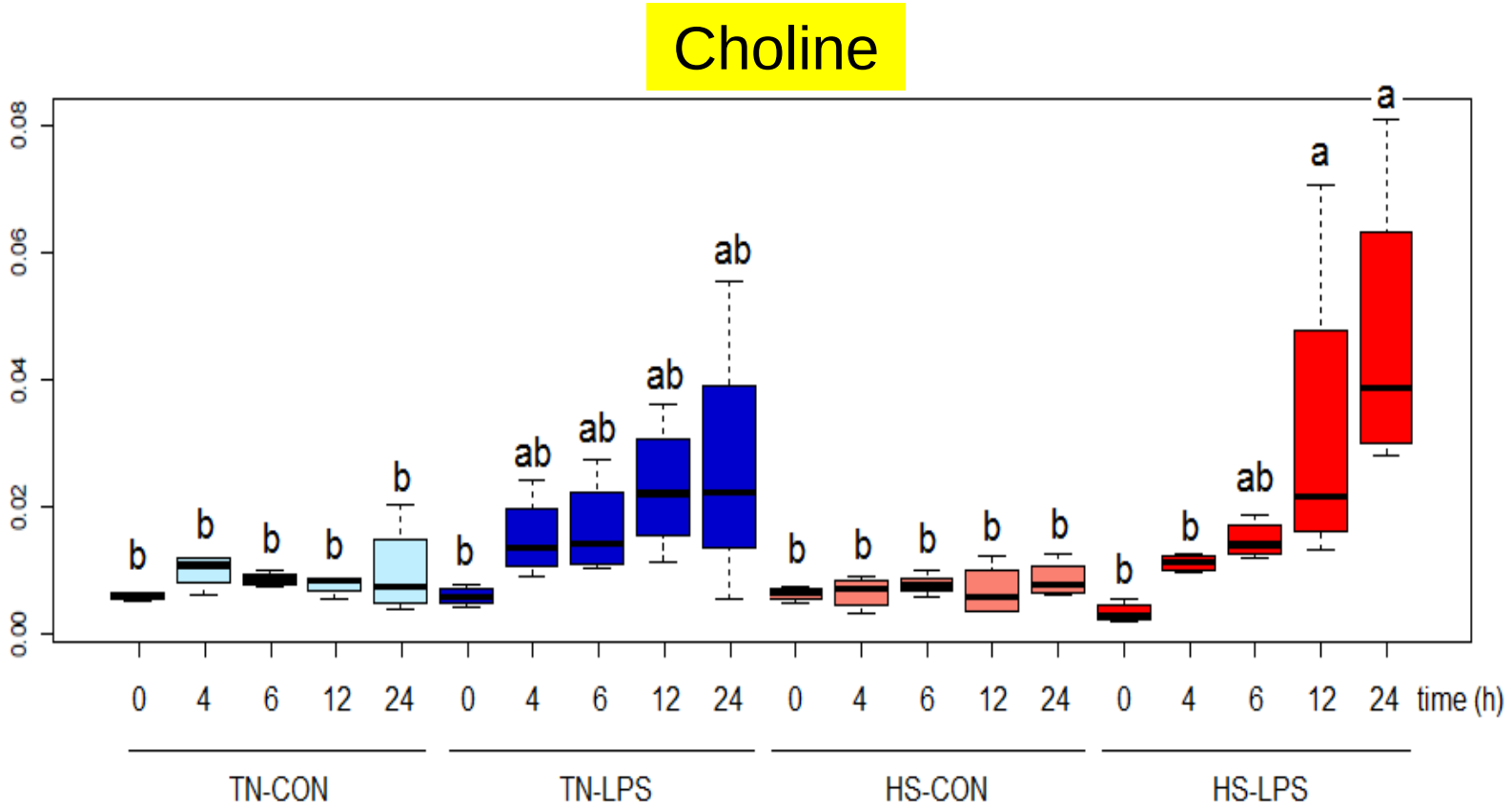


<u>Earlier</u> recovery of the metabolomic profile in TN

CON vs. LPS (inflammation markers)

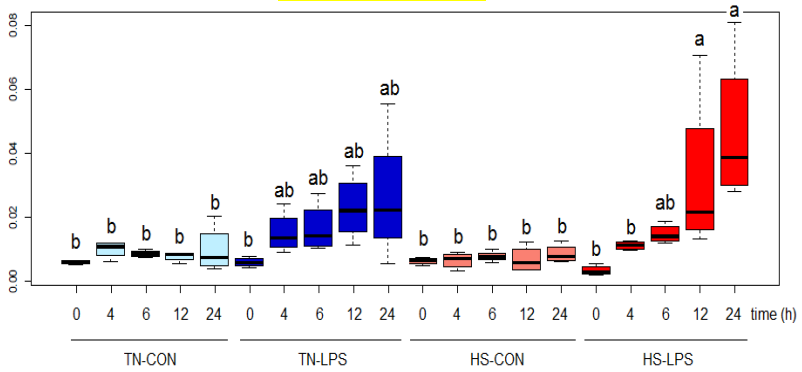
Flux	Chemical shift (ppm)	Metabolite
+	3.19	Choline
+	1.32	L-lactate
+	2.05	N-acetyl CH
+	1.20	Beta-hydroxybutyrate
-	3.84	Lactose
-	4.16	Phosphocholine

Response to LPS (markers of inflammation)

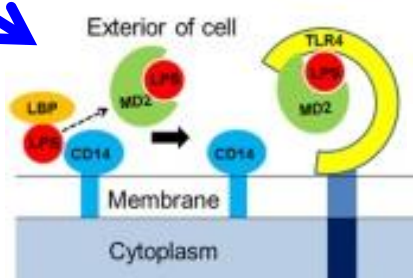


Response to LPS (markers of inflammation)

Choline



Activation of TLR4 receptors by LPS



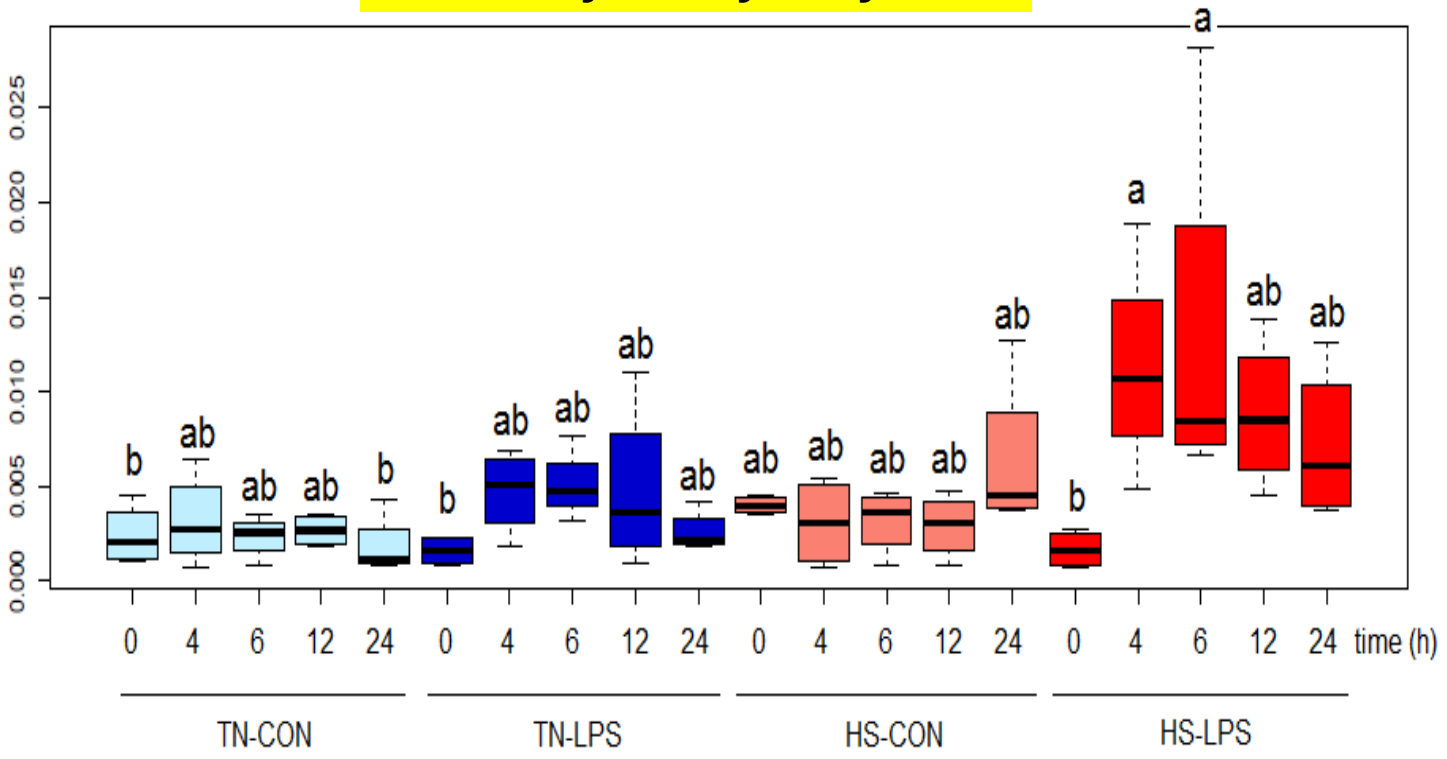
Triggers the cholinergic system

Regulation of pro-inflammatory cytokine production

Awasthi (2014)

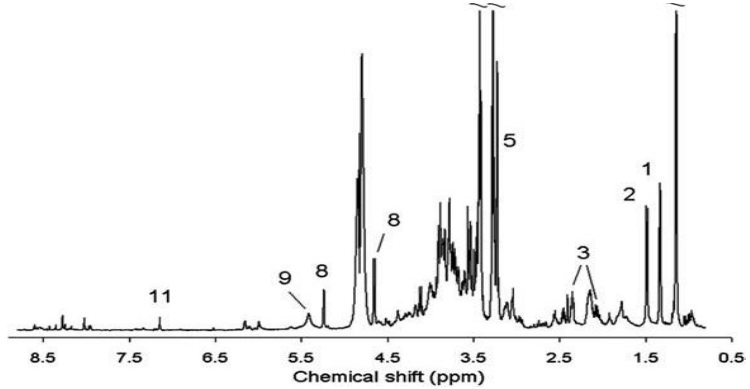
Response to LPS (markers of inflammation)

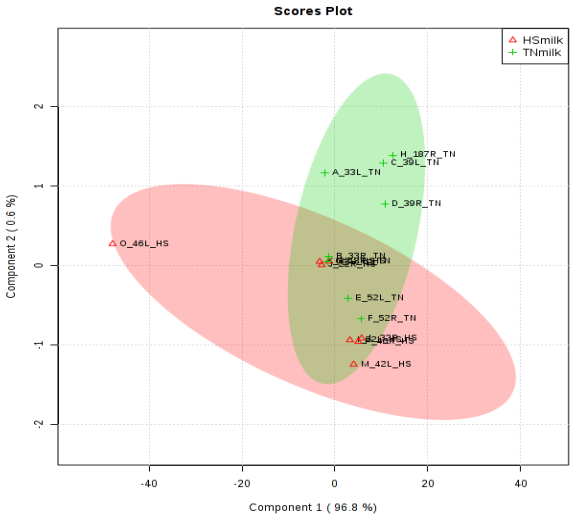
Beta-hydroxybutyrate



Conclusions

- **Milk metabolome** changed throughout time after LPS administration, with **values restored earlier** in TN compared to HS goats.
- Milk **citrate** increased by **heat stress** as part of the heat shock response, enabling the synthesis of pro-inflammatory mediators.
- **Inflammation markers** included **choline**, **phosphocholine**, **N-acetylcarbohydrates**, **L-lactate** and **ß-hydroxybutyrate**.
- The importance of these markers **varied between TN and HS** indicating different mammary immune response.





Thank you for the attention!