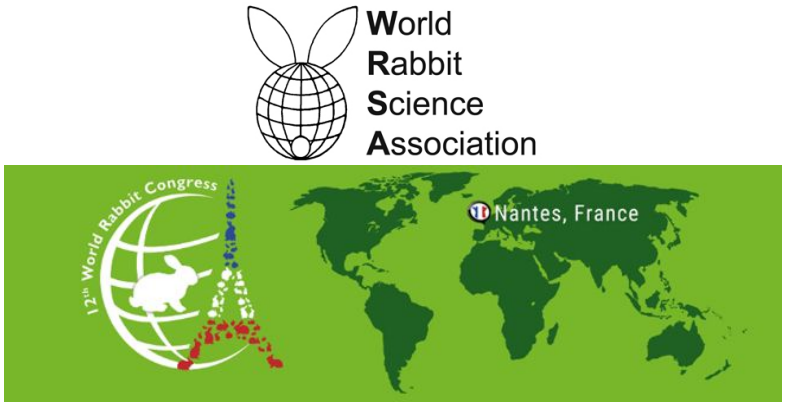

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IMPACT OF MILK COMPOSITION ON NEONATAL MORTALITY IN TWO STRAINS OF RABBITS, THE WHITE POPULATION AND THE SYNTHETIC STRAIN IN ALGERIA

Full text of the communication
+
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IMPACT OF MILK COMPOSITION ON NEONATAL MORTALITY IN TWO STRAINS OF RABBITS, THE WHITE POPULATION AND THE SYNTHETIC STRAIN IN ALGERIA

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ABSTRACT

The aim of our study is to assess mortality of rabbit kits during early life according to the genetic origin of the nursing milk. Therefore, a protocol based on cross-adoptions between litters from the two genetic types of rabbits bred in Algeria, the white population (PB) and the synthetic strain (SS) has been used. Samples of milk were analyzed by liquid chromatography coupled to a mass spectrometer (LC-MS). During three lactations, mortality rates varied according to the genetic origin of the suckled milk. In the control groups, the highest mortality rate was recorded in the litters PB receiving milk PB 18.50 ± 0.18%. This rate drops when these rabbits suckle SS milk (12.50 ± 0.01%, P<0.05). In the control groups, the SS rabbits receiving SS milk, the mortality rate is 12.50 ± 0.03%. This rate increases when SS rabbits suckle PB milk (27.00 ± 0.04%, P<0.05). The different chromatographic profiles highlight a polymorphism of the α_{s1} and α_{s2}-caseins, which is particularly marked in PB milk. The lowest mortality rate is recorded in individuals carrying the natural variant (NV) of α_{s2}-casein, which increases together with the new variant (Var B). The deleterious effect of variant B of a_{s1}-casein (NV/B individuals) seems to be more marked than that of a_{s2}-casein (32% *vs.*15%, P<0.05)), probably due to a cumulative effect of the presence of variant B of a_{s2}-casein. This study reveals significant effects of the genetic origin of milk on the viability of young rabbits, in particular the presence of new genetic variants of the α_{s1} and α_{s2}-caseins.

Key words: rabbit, milk, neonatal mortality, proteins, LC-MS

INTRODUCTION

In Algeria, although local rabbit populations exist and are well adapted to climatic conditions, their prolificacy and weight are too low. A comparison of the reproductive performance of rabbits belonging to two high genetic types bred in the area of Tigzirt, namely the white population of rabbits (PB) and synthetic strain (SS) was performed. The latter demonstrated superiority in terms of weight of female rabbits, prolificacy and born alive at birth (Lebas *et al.*, 2010). However, productivity at weaning in the SS, expressed in number of weaned rabbits per female per litter and/or per year, is very low, especially in summer. These low levels of productivity are related to high mortality during the lactation phase (Zerrouki *et al.*, 2014; Chibah *et al.*, 2014).

In order to identify the causes of this high mortality, studies on the quantitative assessment of the milking function of rabbits were performed, focused on the quantitative assessment (Zerrouki *et al*, 2012; Chibah Aït-Bouziad *et al.*, 2014). The qualitative aspect has poorely been explored, although works on rabbit milk proteins have already been conducted (Dawson *et al.*, 1993; Baranyi *et al.*, 1996; Pak *et al.*, 1999).

The aim of our study was to relate the genetic origin of milk suckled by kits to their viability using cross-adoptions between the litters of PB and SS. The milk samples were analyzed using ESI-Tof LC-MS, a technique combining liquid chromatography and mass spectrometry. This highly resolutive technique, allows the identification and quantification of major milk proteins and their main isoforms resulting from post-translational modifications (Miranda *et al.*, 2020). Our analyzes constitute a solid benchmark for a better understanding of the physiology of lactation in the populations studied and potentially bring up elements of interpretation on the high mortality rate observed during the period of breastfeeding in the PB strain.

MATERIALS AND METHODS

Animals and experimental design

The experiments were conducted over a period of about nine months (from September 2016 to May 2017) in a rabbit farm located in the region of Tigzirt (Northern Algeria), which is characterized by

mediterranean climate (average temperature of 30°C during the day and 23°C at night in summer). Eighty female rabbits belonging to two genetic types, 40 PB and 40 SS were mated with males of the same genetic types and followed during 3 lactation cycles (21 days).
Female rabbits were organized in 4 groups: 2 control groups which nursed their own litters and 2 experimental groups which nursed cross litters (Table1).

Table 1: Organization of cross-adoptions between litters from the PB and SS females

	Groups	Females	Number of lactation cycles	Number of litters	Litter size
Control groups	**Kits PB + Milk PB**	20	3	60	8
	Kits SS + Milk SS	20	3	60	8
Experimental groups	**Kits PB + Milk SS**	20	3	60	8
	Kits SS + Milk PB	20	3	60	8
Total	-	80	-	240	-

PB: White population, SS: Synthetic strain

At parturition, the litters were counted, their size and weight were homogenized to 8 pups/ female and 400 g/litter respectively. Litters were counted each week during 21 days of lactation in order to assess the mortality rate within respective groups.

Chemical Analyses

Milk samples were collected manually from each group on the 10th day of lactation without hormonal stimulation and stocked at -20 °C. The individual milks (n = 80) were diluted with distilled water (1/5 v/v) and skimmed by centrifugation at 2500 g for a period of 20 minutes. Skim milks were then analyzed by LC-MS. Protein separation was carried out on a reverse-phase column (RP-HPLC) using an increasing gradient of acetonitrile in water as previously described by Amroun *et al.* (2015). The identification of milk proteins, on the basis of their molecular weight, required the prior establishment of a database of theoretical molecular weights of female rabbit milk proteins made from a comprehensive literature search, which served as a reference for protein identification from the weights observed in LC-MS.

Statistical Analysis

Data are expressed as means ± SEM. The neonatal mortality was assessed by Student t test. The effect of the protein composition on the mortality of kits was evaluated using the nonparametric Mann-Whitney U test. Significant differences were defined as $P < 0.05$.

RESULTS AND DISCUSSION

Offspring Mortality

Data show that the rate of mortality is significantly different between PB pups/PB milk and SS pups/SS milk groups (18.50 % ± 0.19 % *vs*. 12.50 % ± 0.03 %). In the experimental group, PB pups/SS milk, where the kits are nursed with SS milk the rate of mortality is less important than the control group PB pups/ PB milk (12.50 ± 0.01 % *vs.* 18.50 % ± 0.19 %). However, SS kits nursed with PB milk have a higher mortality rate than the controls (27 ± 0.04 % *vs*. 12.5% ± 0.03) (P <0.001) (Table 2). PB kits survive better when nursed with SS milk compared to the PB group nursed with PB milk (12.50% ± 0.01 *vs.* 18.50% ± 0, 19). However, SS kits nursed with PB milk have a higher mortality rate compared to SS kits receiving SS milk (27.00% ± 0.04 *vs.* 12.50% ± 0.03).

Table 2: Evaluation of the mortality rates in the control and experimental groups of rabbits

Litter/Milk	Females	Kits at D1	Weight kits at D21	*P*	Mortality (%)	*P*
PB/PB	20	480	391,20 ± 2,09^{c}	*<0,001*	18,50 ± 0,18^{c}	*<0.001*
SS/SS	20	480	420,00 ± 1,23^{b}	*<0,001*	12,50 ± 0,03^{b}	*<0,001*
PB/SS	20	480	420,00 ± 1,11^{b}	*<0,001*	12,50 ± 0,01^{b}	*<0,001*
SS/PB	20	480	350,40 ± 3,41^{a}	*<0,0001*	27,00 ± 0,04^{a}	*<0,001*

** (a, b) are significatively différent (p<0.05); D1: 1st Day of lactaction, D21:21st Day of lactaction*

Existence of new variants of major lactoproteins

The comparison of the chromatograms of milks from the 2 populations PB and SS (n = 80), shows a heterogeneity of the PB population compared to the SS population. Although no quantitative differences between the lactoproteins of the two strains are observed, major qualitative differences can nevertheless be noticed between SS and PB, suggesting the existence of genetic polymorphisms of major proteins, in particular at the level of α_{s2} and α_{s1} –caseins. Indeed, two new variants have been identified for α_{s2} - casein (Variants B and C) and a new variant for α_{s1}-casein (Variant B). Moreover, LC-MS analyses allowed the determination of the masses of the new variants B of α_{s2} and α_{s1} –caseins (Figure 1 and Table 3) and of a new variant C (21207.18 Da, data not shown).

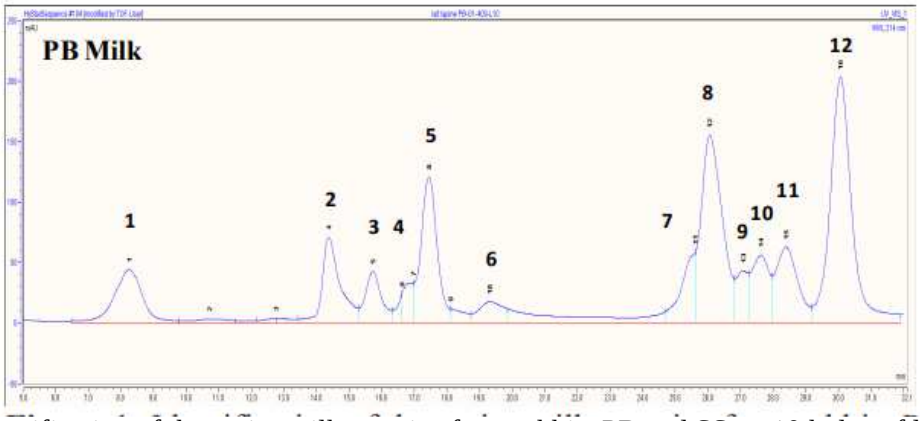

Figure 1: Identification of the major milk proteins from rabbits PB and SS on 10th day of lactation. Peaks 1: glycosylated κ -cas, 2: Lactoferrin, 3: αs2 -cas, 4-5: WAP (Whey Acidic Protein), 6: α-lactalbumin + Serumalbumin, 7-8: αs2-like cas, 9- 11: αs1 -cas, 12: β –cas

Table 3: Determination of genetic variants of major milk lactoproteins in PB strain

	Mass (Da)	Signal Intensity	Natural Variants	Theoretical Mass (Da)	Putative New Variants
Peak 1			glycosylated κ -cas		
Peak 2	76803.04	4437	Lactoferrin VN -10P	75685.97	
Peak 3	20246.89	4529	α_{s2} –cas VN -4P	20246.5715	
Peak 4	11680.95	2746	WAP VN -1P	11681.4218	
Peak 5	11761.37	19508	WAP VN -2P	11761.4018	
	21134.61	1335			α_{s2} –cas VB -4P
Peak 6	66070.14	902	SA VN -1P	66095.39	
Peak 7	20125.43	1250	α_{s2}-like cas VN -6P	20126.2687	
Peak 8	20205.81	15050	α_{s2}-like cas VN -7P	20206.2387	
	24404.26	4008	α_{s1} –cas VN (-1Q) -7P	24403.7316	
	24533.15	6417	α_{s1} –cas VN -7P	24531.8616	
Peak 9	24321.68	533	α_{s1} –cas VN (-1Q) -6P	24323.7526	
	24452.08	541	α_{s1} –cas VN -6P	24451.8826	
Peak 10	24289.15	1577			α_{s1} –cas VB -7P (-1Q)
	24417.41	1813			α_{s1} –cas VB -7P
Peak 11	24209.27	3305			α_{s1} –cas VB -6P (-1Q)
	24337.16	3406			α_{s1} –cas VB -6P
Peak 12	24856.58	57796	β VN -4P	24855.874	

VN: Natural variant; VB : New variant

Correlation between mortality and α_{s2} -casein variants in individuals of the PB type

A correlation was observed between the presence of variants B or C of α_{s2} -casein and the mortality in the PB rabbits (Table 4). The mortality is higher in animals homozygous (VB/VB) than in rabbits heterozygous (VB/VN) and (VB/VC). The presence of VC causes a deleterious effect, but less marked than that of VB. Results within homozygous genotype (VN/VN) corresponds to a normal mortality rate in a standard breeding population. However, it should be noted that the simultaneous presence of VN and VB, results in a strong reduction in the deleterious effect of the milk.

Table 4: Correlation between pup mortality and presence of α_{s2}-casein variants in PB milk

	% Mortality (M. ± Sem)	VB/VB 54.05 ± 2.95	VN/VB 15.35 ± 1.82	VN/VN 11.65 ± 1.34	VB/VC 38.38 ± 4.46
VB/VB	54.05 ± 2.95		S[b]	S[b]	S[b]
VN/VB	15.35 ± 1.82			NS[a]	S[b]
VN/VN	11.65 ± 1.34				S[b]
VB/VC	38.38 ± 4.46				

VN: Natural Variant; VB: New Variant B; VC: New Variant C, S: significative différence ; NS: non significative différence; [a]: Student test ; [b]: Mann-Whitney test

Correlation between mortality and α_{S1} -casein variants in individuals of the PB type

We also observed a positive correlation between the neonatal mortality of PB rabbits and the presence of α_{s1} -casein VB (Table 5). However, the deleterious effect seems less marked than that of the α_{s2} -casein VB (respectively 31.89 ± 6.21 and 54.05 ± 2.95).

Table 5: Statistical tests between mortality and variants of α_{s2}-casein in PB

	% Mortality (M. ± SEM)	VN/B	VN/VN
		31.89 ± 6.21	12.27 ± 1.10
VN/B	31.89 ± 6.21		S^b
VN/VN	12.27 ± 1.10		

VN : Natural Variant; VB: New Variant B ; S^b: significative différence (Student t test)

CONCLUSION

The results obtained during this work made it possible to improve our knowledge on the dairy aptitudes of the rabbits currently raised in Algeria, in this case the white population (PB) and the synthetic strain (SS). Moreover, cross-adoption experiments demonstrated the involvement of milk in the neonate mortality during lactation period. Analysis of the milk protein fraction by LC-MS, revealed the existence of new genetic variants of the α_{s2} and α_{s1}-caseins correlated with the high mortality rate in rabbits of the PB type. Comparison of mortality rates in rabbits fed with milks containing the two a_{s2}-casein variants, suggest that VN/B a_{s2}-casein variant may constitute one of the elements which are believed to be responsible for the high mortality rates observed within PB litters.

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12th World Rabbit Congress
November 3-5 2021 – Nantes France

Impact of milk composition on neonatal mortality in two strains of rabbits, the white population and the synthetic strain in Algeria

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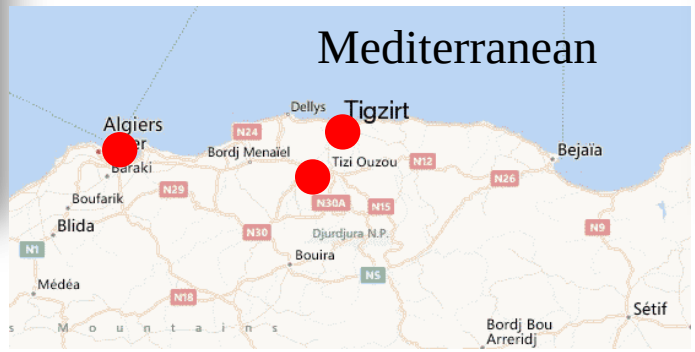
GABI Génétique Animale & Biologie Intégrative

INRAE

Strains PB and SS

White Population (PB)

Imported hybrides Hyplus



Synthetic strain (SS)

Local Population x **INRA2666**

	Number of animals		Mean ± SEM	
Strains	**SS**	**PB**	**SS**	**PB**
Born alive	104	114	9 ± 3.3	8 ± 3.6
Weaned	94	100	8 ± 1.6	7 ± 1.6
Mortality between birth and weaning (%)	94	100	**11.32 ± 0.2**	**18,03 ± 0.2**

- **At birth: no difference between the two strains**
- **At weaning: higher mortality rate in PB**

In vivo approach

80 females
+
3 Lactation cycles
+
Homogeneization at birth

Females SS

CROSS-FOSTERING
between PB and SS litters

Females PB

Evaluation of the mortality rates

Litter / Milk	Females	Pups on PND1	Mortality (%)	*P*
PB / PB	20	480	**18.50 ± 0.18**	***<0.001***
SS / SS	20	480	**12.50 ± 0.03**	***<0.001***
PB / SS	20	480	**12.50 ± 0.01**	***<0.001***
SS / PB	20	480	**27.00 ± 0.04**	***<0.001***

➢ **Milk composition may be a major factor, contributing in neonatal mortality of PB rabbits**

Biochemical approach

Working hypotheses

Do the constituents of milk play a role in neonatal mortality?

Protein composition?

- Significant polymorphism of lactolactoproteins
- Existence of genetic variants

Baranyi *et al.*, 1995
Hiripi *et al.*,1998
Devinoy *et al.*, 1999
Bolet *et al.*, 2007

Consequences on pups' mortality

Bolet *et al.*, 2007:
Role of κ-casein polymorphism in *in utero* mortality

- **What about the role of other caseins' polymorphisms in neonatal mortality?**

PRINCIPLES OF THE LC-MS METHOD

Liquide chromatography mass spectrometry (Miranda et *al.*, 2013)

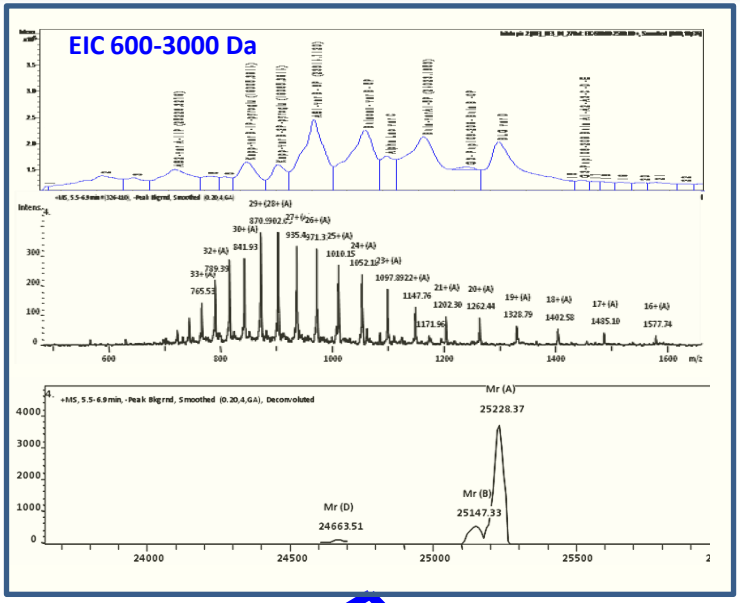
Ultimate 3000

Thermo SCIENTIFIC

microTOF focus

BRUKER

Milk → RP-HPLC → MS (ESI-TOF)



(EIC)
Extracted Ion Chromatogram

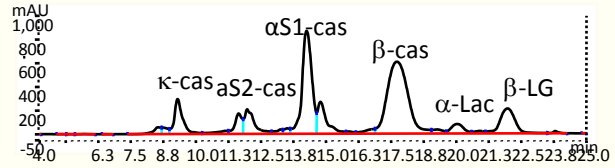
↓

Spectre des ions multichargés

↓

Spectre de masse déconvolué

UV 214nm chromatogram (bovine milk)



Quantification using UV signal

(% of the chromatogram pics total area)

Quantification using the mass signal intensity

Observed masses

Multi species
Theoretical masses

Data Bases

Bovine (3000)
Ovine (1700)
Caprine (2000)
…. Rabbit

« Classical » method

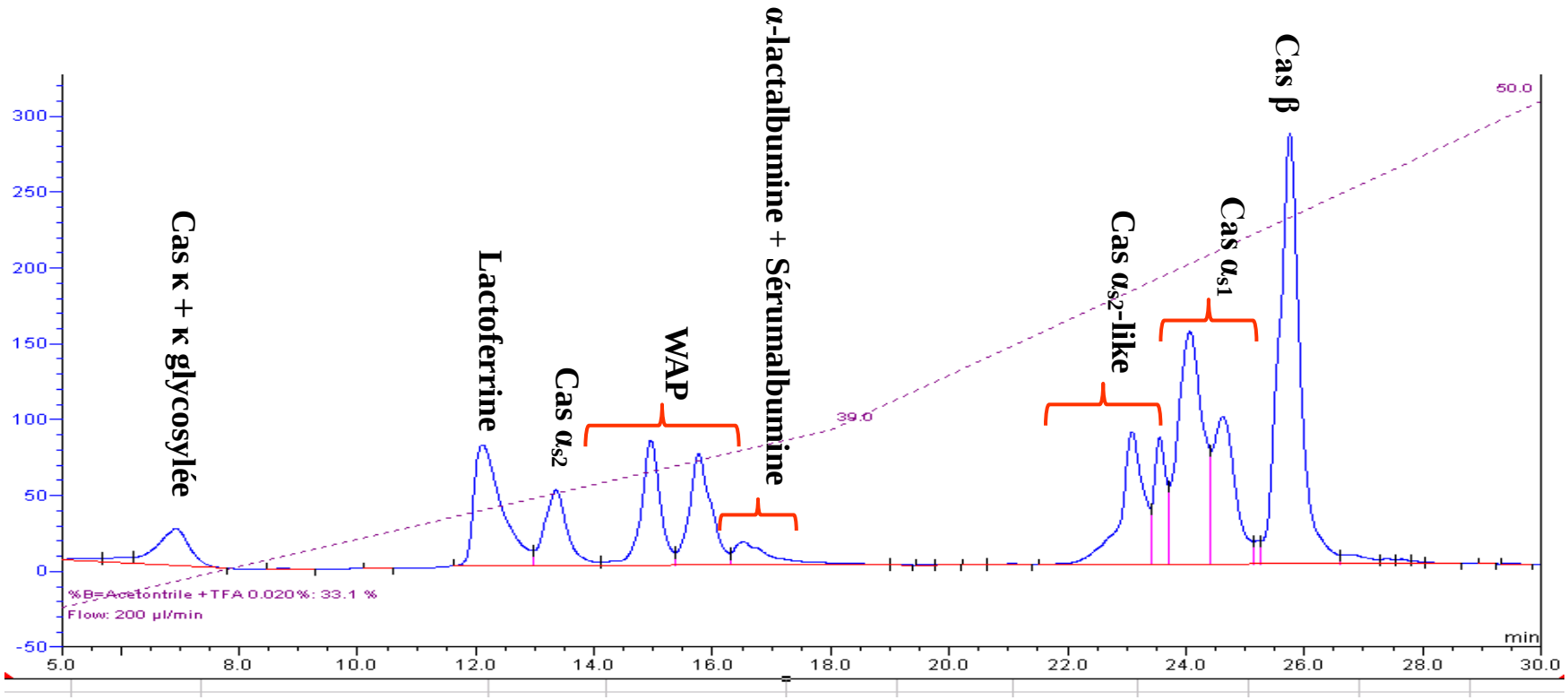
“New method”

IDENTIFICATION of Protein Isoforms

QUANTIFICATION

splicing variants, genetic variants, post-translational modifications (glycosylation, phosphorylation), proteolysis products

➢Identification of the major lactoproteins by LC-MS



➢**Identification of the 5 caseins: κ, α_{s2}, α_{s2} -like, α_{s21} and β**

➢ **Identification of the 4 major serum proteins:**

➢ **lactoferrin, WAP, α-lactalbumin and serum albumin**

➢**Some lactoproteins have a double peak: WAP, α_{s2} -like, α_{s1} (phosphorylation isoforms)**

Determination of major lactoproteins' variants in PB (LC-MS)

Major lactoproteins	Variants	Theoretical Mass (Da)	Observed Mass (Da)
κ-cas	VN	18 018.56	
α_{s2}-cas	VN	19 926.66	
	V B		**20 813.08**
	V C		**20 887.08**
WAP	VN		
α_{s2}-like-cas	VN	19 646.38	
	VB		
α_{s1}-cas	VN	23 972.01	
	VB		**23 856.92**
β-cas	VN	24 535.95	

➢ **Two new α_{s2}-casein variants: VB and VC**

α_{s2}-cas VN 4P
20 246, 89 Da

→

α_{s2}-cas VB 4P
21 134,61 Da

→

α_{s2}-cas VC 4P
21 207,18 Da

+887,72 Da (supplementary exon?) **Δm -72,8 Da**

➢ **One new α_{s1}-casein variant: VB**

α_{s1}-cas VN 7P
24 531,86 Da

→

α_{s1}-cas VB 7P
24 417,41 Da

Δm -145,45 Da

Correlation between the nature of variants and neonatal mortality in PB (I)

α_{s2}-casein variants

4 groups: homozygotes VB/VB and VN/VN; heterozygotes VN/VB and VB/VC

	% Mortality (M. ± SEM)	VB/VB 54.05 ± 2.95	VN/VB 15.35 ± 1.82	VN/VN 11.65 ± 1.34	VB/VC 38.38 ± 4.46
VB/VB	54.05 ± 2.95		S	S	S
VN/VB	15.35 ± 1.82			NS	S
VN/VN	11.65 ± 1.34				S
VB/VC	38.38 ± 4.46				

- **The lowest mortality rate is observed in individuals carrying the natural variant (VN / VN = VN / VB)**
- **Neonatal mortality increases with the presence of new variants: VB and VC**
- **Mortality rate: (VB / VB)> (VB / VC)> (VN / VB)**

Correlation between the nature of variants and neonatal mortality in PB (II)

α_{s1}-casein variants

2 groups: VN/VN et VN/VB

	% Mortality (M± SEM)	VN/B	VN/VN
		31.89 ± 6.21	12.27 ± 1.10
VN/VB	31.89 ± 6.21		S
VN/VN	12.27 ± 1.10		

- **Lowest mortality rate: individuals carrying the natural variant VN**
- **Neonatal mortality: increases with the presence of the new variant VB**
- **In heterozygotes, the deleterious effect of α_{s1}-casein VB variant is more important than that of α_{s2}-casein VB variant (32% *vs.* 15%)**

- **The nature of the genetic variants of α_{s2} and α_{s1} caseins impacts neonatal mortality in PB rabbits**

Conclusions

- This work has demonstrated the major role played by casein composition in milk for the neonatal health of the pups in the rabbit.

- It has also demonstrated the importance of genetic polymorphisms, in particular those of α_{s1}- and α_{s2}-caseins, in rabbit neonatal mortality.

- Therefore, the question of the biological function of α_{s1}- and α_{s2}-caseins can be raised;

    - Bovine α_{s2}-casein = carrier of peptides with anti-microbial activity? (Alvarez-Ordóñez et al., 2013)
    - New variants: loss of this activity?

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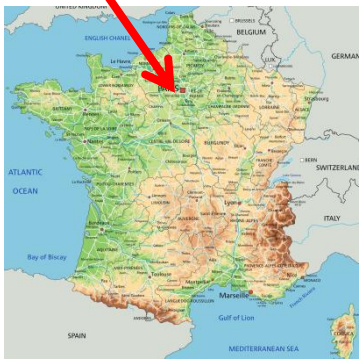

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