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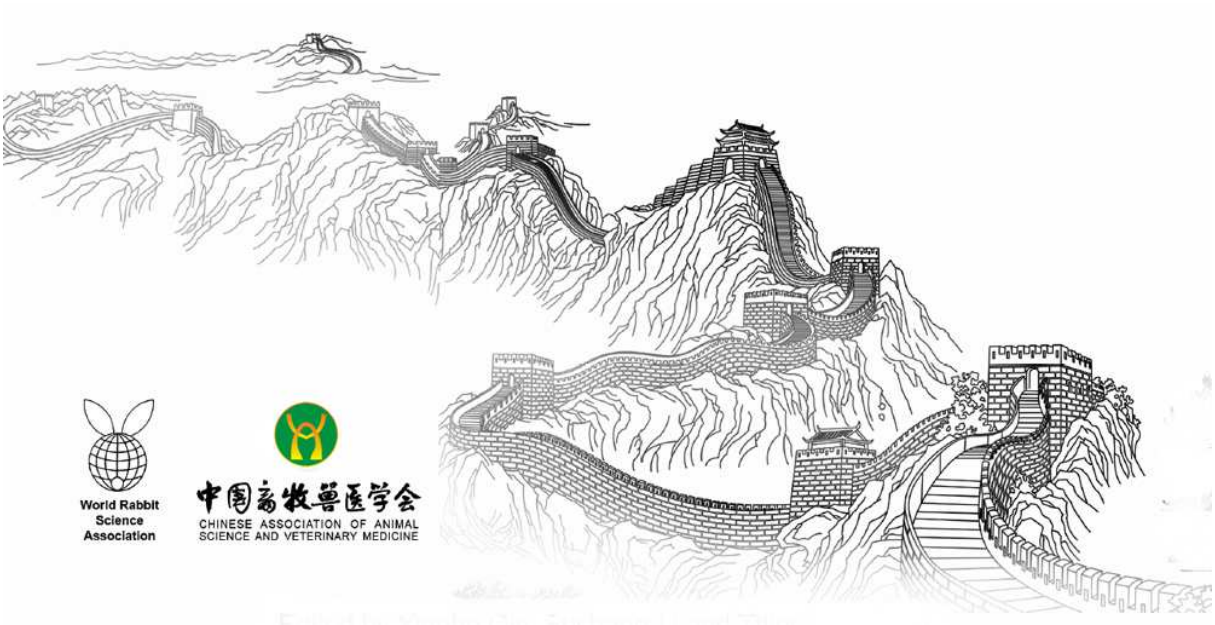

CHARACTERIZATION OF THE PROTEIN FRACTION OF MILK PRODUCED BY TWO GENETIC TYPES OF RABBITS IN THE REGION OF TIZI OUZOU

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ABSTRACT

The objective of the study was to analyze milk protein composition (major proteins) of two genetic types of rabbits bred in Tizi-Ouzou (Algeria): the white population (PB) and the "synthetic" strain (SS). Differences in size and litter weight at weaning were observed amongst the two types, which might be due to qualitative and/or quantitative variations in milk composition. Milk samples (n=7) from dams of the two genetic types were collected (10th day of lactation) and analyzed by means of liquid chromatography associated to a mass spectrometer (LC-MS). We were thus able to identify and characterize the major milk proteins in both types of rabbits and determine their post-translational modification isoforms. Alpha-s_1 and β represented 50% of the total milk proteins; α_{s2}-like-, α_{s2}- and κ- caseins represented respectively 13.5, 4 and 2.7%. Whey Acidic Protein was the main whey protein (14.5%) while lactoferrin represented 10% of the total milk proteins. Chromatographic profiles of milk proteins were similar between the two genetic types of rabbits. In the "synthetic" strain, a strong dispersal around the mean of the relative proportions of α_{s2} casein and the mix α-Lac + SA was observed ($CV\alpha_{s2}$ = 0.24 in PB *vs.* 0.46 in SS and CVα-Lac + SA = 0.19 *vs.* 0.274 in PB and in SS respectively).

Keys words: LC-MS, Milk, Rabbit, Protein

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 made. 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 Aït-Bouziad et al, 2014).

In order to identify the causes of this high mortality, studies on the quantitative assessment of the milky function of rabbits were performed. They focused on the quantitative assessment of the milky function of rabbits (Zerrouki et al, 2012; Chibah Aït-Bouziad et al, 2014). The qualitative aspect (detailed analysis of the composition of milk) has however never been explored. Works on rabbit milk proteins have already been conducted (Dawson et al, 1993; Baranyi et al, 1996; Pak et al, 1999). Yet, these studies focus on the characterization of the primary sequences of milk proteins.

The aim of our study was to identify and characterize the major protein fraction of milk from rabbits belonging to two Algerian genetic types using an analytical technique that calls for the coupling of chromatography in liquid phase and mass spectrometry of the type ESI-Tof (LC-MS). This highly resolute technique, which is reliable and robust, allows the identification and a quantification of major milk proteins and their main isoforms resulting from post-translational modification: glycosylation and phosphorylation (Miranda et al, 2013). Our analyzes will constitute a solid benchmark for a better understanding of the

physiology of lactation in the populations studied and potentially bring about elements of interpretation on the high mortality rate observed during the period of breastfeeding in the SS strain.

MATERIALS AND METHODS

The experiment was conducted over a period of about nine months (from June 2013 to February 2014) in a rabbit farm located in the region of Tigzirt (Tizi Ouzou, Northern Algeria) wich is characterized by mediterannen climate (average temperature of 30°C during the day and 23°C at night in summer).

Seven multiparous female rabbits belonging to two genetic types - three of them from the white rabbit population (PB) and four from the synthetic strain (SS) - were mated with males of the same genetic types and followed during a lactation cycle (21 days). At parturation, the litters were counted, weighed and equalized to 8 young rabbits per female. Milk samples were collected manually (10^{th} day of lactation) without hormonal stimulation. The milk collected during the summer was supplemented with Thimerosal (5%) before being frozen at -20 °C.

The analysis was performed on the milk samples taken during the second week of lactation. The individual milks (n = 7) was 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 means of 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 Saadaoui et al (2014).
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. It served as a reference for protein identification from the weights observed in LC-MS. The relative quantification of major families of milk proteins (caseins and whey proteins) was carried out by means of integration of the peak areas of the chromatogram (Optical Density at 214 nm). Values are expressed in percentage of the total area of the peaks of the chromatogram. Statistical test of Mann-Whitney was used.

RESULTS AND DISCUSSION

Identification of major proteins of milks from rabbits of genetic types PB and SS

An analysis by means of LC-MS of each individual milk allowed the identification of the caseins κ, α_{s2}, α_{s2}-like, α_{s1}, and β respectively corresponding to peaks A, C, F (F_1 and F_2), G (G_1 and G_2) and H. The serum proteins are also identified : lactoferrin, WAP (Whey Acidic Protein), α-Lactalalbumin and serum albumin corresponding to peaks B, D (D_1 and D_2) and E respectively (figure 1).

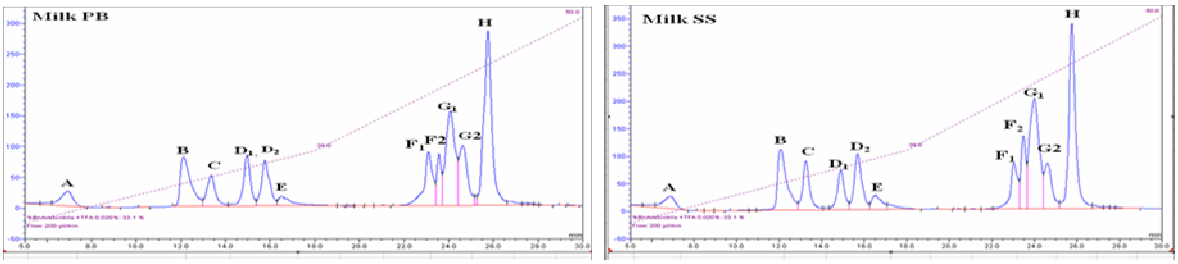

Figure 1: Identification of the major milk proteins from rabbits PB and SS.
Peak A**:** Cas κ + κ glycosylated, peak B: Lactoferrin, peak C: Cas α_{s2}, peaks D_1 and D_2: WAP, peak E:α-lactalbumin + Serumalbumine , peaks F_1 and F_2: Cas α_{s2}-like , peaks G_1 and G_2: Cas α_{s1}, peak H: Cas β

In both genetic types of milk, the WAP, α_{s2}-like and α_{s1} casein are present in the form of two peaks corresponding to different isoforms of phosphorylation: D_1 (WAP 1P), D2 (WAP-2P), F_1 (Cas α_{s2}-like-2 to 6P), F_2 (Cas α_{s2}-like-7P), G_1 (Cas α_{s1}-6P), G_2 (Cas α_{s1}-7P) (figure 2).

As for the α-lactalbumin and serum albumin, they are co-eluted (peak E). It should also be noted that the molecular weights observed for the various milk proteins on the two types of milk do not differ and that they correspond to those referenced in the protein databases (Uniprot, http://www.expasy.org/).

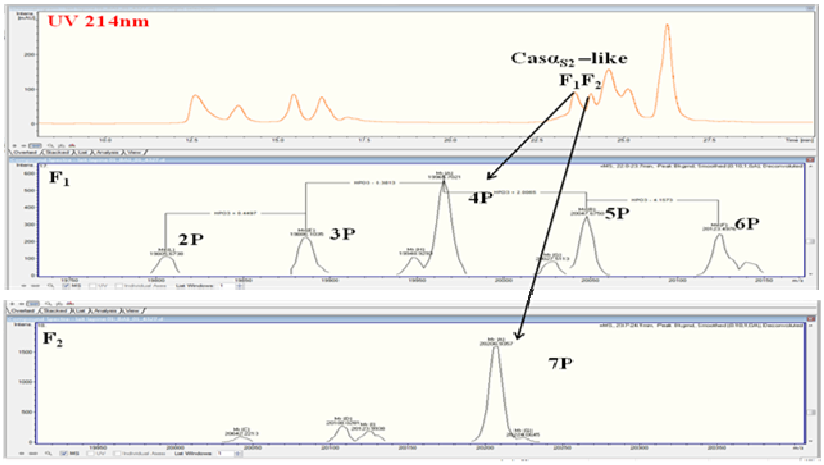

Figure 2: α_{s2}-like casein phosphorylation isoforms in the PB and SS types of milk

Analysis of chromatographic profiles of milks of PB and SS types

The analysis of LC-MS chromatographic profiles allows us to observe a relative homogeneity of the profiles within the same population and between populations but with some differences in intensity of certain peaks. These differences, particularly visible at the α_{s2} casein (peak C) and α-Lac mixture + SA (peak E). This fact, suggests the existence of a higher variability of relative proportions of certain milk proteins in the SS group (figure 3).

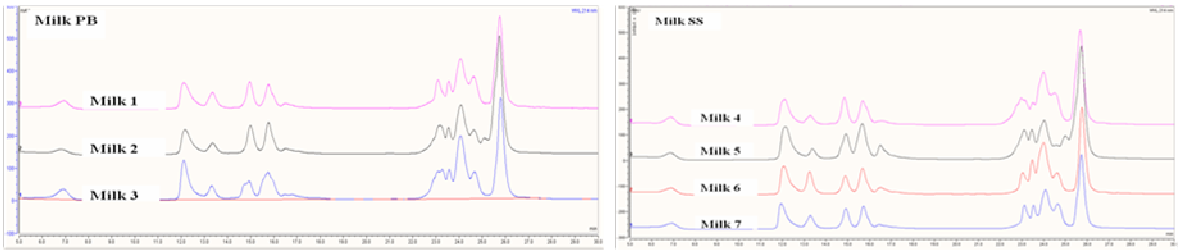

Figure 3: Chromatographic profiles of the milk of PB type (samples 1, 2, 3) and SS type (samples 4, 5, 6, 7)

Quantification of the major types of proteins in milk of two genetic rabbits (PB and SS)

The two genetic types show no significant difference when comparing the mean of the relative proportions of each of the major milk proteins (statistical test of Mann-Whitney). However, there was greater dispersion around the mean in the case of α_{s2} casein, α_{s1}, β and α-Lac mixture + SA in individuals of the SS genetic type ($CV\alpha_{s1}$ = 2.25 in SS *vs* 0.09 in PB, $CV\alpha_{s2}$ = 0.457 in SS *vs* 0.239 in PB, CV β = 2.22 inSS *vs* 0.12 in PB and CV α-Lac + SA = 0.274 in SS *vs* 0.190 in PB) (table.1).

Table 1: Relative quantification (% of total peaks in the chromatogram) of the major milk proteins in the rabbit milk from two genetic types (PB n = 3 and SS n = 4)

	Caseines						SP			Other peaks	Total
	κ	α_{s2}	α_{s2}-like	α_{s1}	β	Tolal Cas	LF	WAP	a-Lac + SA	Total SP		
Type PB	2.9 ± 0.25	3.8 ± 0.17	13.8 ± 0.06	23.3 ± 0.09	26.9 ± 0.12	70.6± 0.01	9.6 ± 0.08	14.7 ± 0.73	2.2 ± 0.13	26.5 ± 0.06	2.9 ± 0.28	100
Type SS	2.4 ± 0.29	4.2 ± 1.12	13.3 ± 0.58	24,1 ± 2,25	26.4 ± 2.22	70.4± 2.22	10.4 ± 0.50	14.4 ± 0.80	2.8 ± 0.45	27.6 ± 1.61	2.0 ± 0.44	100

The showed values correspond to mean ± SEM, LF: Lactoferrine, SP: serum proteins

The major milk proteins have identical molecular in the milk of both genetic types of animals and correspond to those described in the literature. On the chromatograms obtained by RP-HPLC, the α_{s2}, α_{s2} -like caseins and the WAP are present in the form of two peaks corresponding to different degrees of phosphorylation. Baranyi et al (1995) have already reported the presence of multiple isoforms of phosphorylation for different casein and WAP. Through our methodological approach (LC-MS), we have not only confirmed these results, but we have also accurately determined the extent of phosphorylation of different isoforms.

The α_{s1} and β caseins represent 50% of total caseins. While the α_{s2}-like casein is three to four times more abundant (13.5%) than α_{s2} and κ caseins (respectively 4 and 2.7% in average). Among the whey proteins the WAP is majority (14.5%), as described by Baranyi et al (1995). Lactoferrin is abundantly represented in the rabbit milk (10%) compared to the milk of other species (about 1% in goats and cattle). This molecule is also present in huge quantities in breast milk (15% ; Martin and Grosclaude, 1993) and camelids (22%; Kappeler, 1998). It is worth noting that the ratio casein/soluble proteins in rabbit milk (70/30) is different from that observed in ruminants (cattle 80/20) or women (40/60) (Martin and Grosclaude, 1993).

CONCLUSION

The preliminary results presented here on rabbit milk analysis of two different genetic types (PB and SS) by liquid chromatography coupled to a weight spectrometer of the ESI-TOF type, demonstrate the potential of this method which allows us to have access to a detailed description of the composition of the "major proteins" fraction of rabbit milk. The major milk proteins, as well as their main isoforms resulting from post-translational modifications, have been highlighted. These analyses suggest a significant variability in the relative proportions of milk proteins in the SS population that could be a start point of research to determine the differences on variability during lactation. These results are to be confirmed, and a study is underway to validate them on a larger sample. Chromatographic profiles of milk proteins were similar between the two genetic types of rabbits. In the "synthetic" strain, a strong dispersal around the mean of the relative proportions of α_{s1},α_{s2}, ,β casein and the mix α-Lac + SA is observed ($CV\alpha_{s1}$ = 2.25 in SS *vs* 0.09 in PB, $CV\alpha_{s2}$ = 0.457 in SS *vs* 0.239 in PB, CV β = 2.22 in SS *vs* 0.12 in PB and CV α-Lac + SA = 0.274 in SS *vs* 0.190 in PB).

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PROTEIC FRACTION ANALYSIS OF MILK PRODUCED BY TWO GENETIC TYPES OF RABBIT DOES: SYNTHETIC LINE AND WHITE POPULATION, RAISED IN THE REGION OF TIZI OUZOU (ALGERIA)

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BACKGROUND

❑ Neonatal feeding depends exclusively on maternal milk production; indeed maternal milk represents the first nutritional support during the first days of life.

In sufficient quantity during lactation, it allows harmonious growth of young rabbits (Fortun-Lamothe and Gidenne, 2003).

❑ In Algeria, the introduction of a "synthetic" strain (SS) and its dissemination through the farms has improved the adult weight and prolificity (Gacem and Bolet, 2005; Gacem *et al*, 2008).

❑ Rabbit milk is characterized by a high protein content (x6 and x20 as compared with bovine and human milks, respectively), the absence of β-lactoglobulin, the presence of whey acidic protein (WAP) and a second α_{s2} casein (α_{s2}-like) as well as a high concentration of lactoferrin (Martin *et al*, 2011). Rabbit milk proteins have been essentially characterized at the cDNA levels (Schaerer *et al*, 1988; Devinoy et al., 1988; Bösze *et al*, 1993) and at the amino acid sequence (Dawson *et al*, 1993; Baranyi *et al*, 1996; Pak *et al*, 1999).

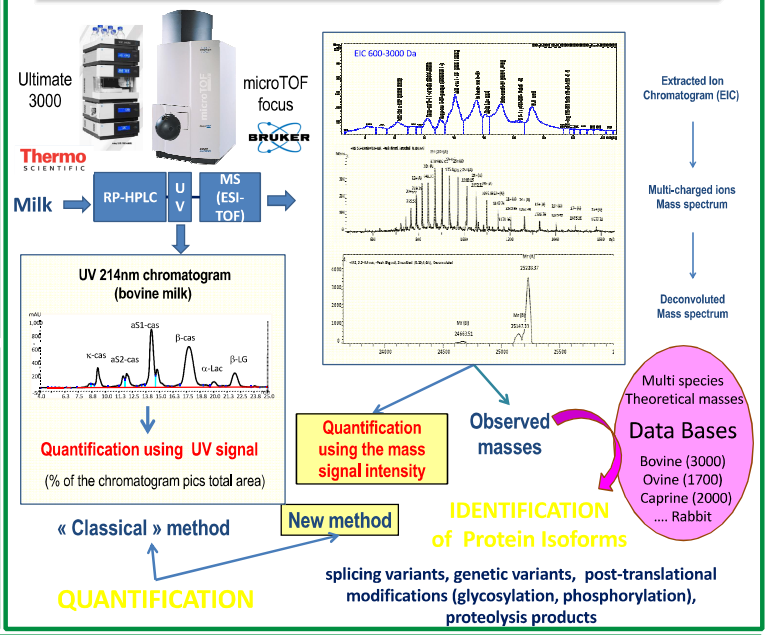
OBJECTIVES

❑ The objective of the present study was to analyze milk protein composition (major proteins) of two genetic types in Tizi-Ouzou (Algeria): the white population (PB) and the "synthetic" strain (SS).

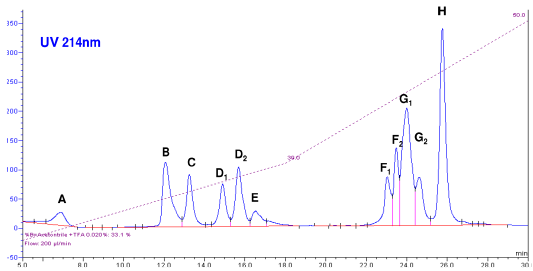
❑ Using a powerful LC-MS method (Miranda *et al*, 2014) initially implemented on cattle, we profiled milk proteins, including their multiple isoforms (genetic and splicing variants, post-translational modifications) in order to:

1) **Identify and characterize the major milk proteins in rabbit milk.**
2) **Compare the protein composition (qualitatively and quantitatively) in milks from dams belonging to the SS strain and from dams belonging to the PB strain.**

PRINCIPLES OF THE LC-MS METHOD



RP-HPLC chromatogram of individual rabbit skimmed milk (NZ-INRA_1077)



A = κ-Csn (non glycosylated + glycosylated), **B** = Lactoferrin, **C**= α_{s2}-Csn (2 et 3P- isoforms), **D1**= WAP 1P-isoform, **D2** = WAP 2P-isoform, **E**= α-Lac + Serum Albumin (SA), **F1**= α_{s2}-Csn-like (2 to 6P-isoforms), **F2**= α_{s2}-Csn-like (7P-isoform), **G1**= α_{s1}-Csn (6P-isoform); **G2**= α_{s1}-Csn (7P-isoform), **H**= β-Csn (1 to 4P-isoforms)

IDENTIFICATION AND CHARACTERIZATION OF THE MAIN RABBIT MILK PROTEINS

➢ **Construction of a database** gathering all **theoretical molecular masses** collected from the literature and the UniprotKB database.

➢ **Identification of peaks corresponding to the major milk proteins in rabbit milk:** κ, α_{s1}, α_{s2}, α_{s2}-like and β-caseins (Csn), Whey Acidic Protein (WAP), α-lactalbumin, lactoferrin and serum albumin (SA); determination of their molecular weights as well as those of their main phosphorylation isoforms.

✓No significant difference observed between the 2 strains.
✓Type SS : higher dispersion around the mean (α_{s2}, α_{s2} and β-Csn, α-Lac + SA)

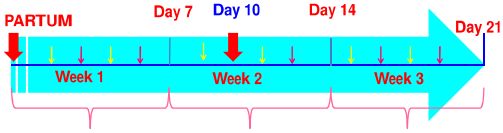
Relative proportions of the main milk proteins
(% of total peaks of the chromatogram)

		PB Type	SS Type
Caseins	κ	2,9 ± 0,25	2,4 ± 0,29
	α_{s2}	3,8 ± 0,17	4,2 ± 1,12
	α_{s2}-like	13,8 ± 0,06	13,3 ± 0,58
	α_{s1}	23,3 ± 0,09	24,1 ± 2,25
	β	26,9 ± 0,12	26,4 ± 2,22
Total caseins		70,6 ± 0,01	70,4 ± 1,36
Serum Proteins (SP)	Lactoferrin	9,6 ± 0,08	10,4 ± 0,50
	WAP	14,7 ± 0,73	14,4 ± 0,80
	α-Lac + SA	2,2 ± 0,13	2,8 ± 0,45
Total SP		26,5 ± 0,06	27,6 ± 1,61
Other peaks		2,9 ± 0,28	2,0 ± 0,44
Total		100	100

EXPERIMENTAL DESIGN

➢ Seven multiparous female rabbits belonging to the two genetic strains, the white population (n=3) and the synthetic strain (n=4), were mated with males of the same genetic strains and followed during a lactation cycle (21 days).

➢ Milk samples were taken manually (without hormonal stimulation) twice a week during the three weeks of lactation. The milk collected during the summer was supplemented with Thiomersal (5%) before being frozen at -20 °C.

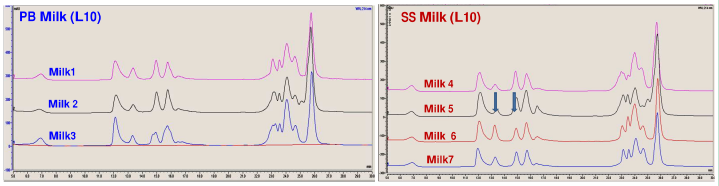


Milk collection (L10)

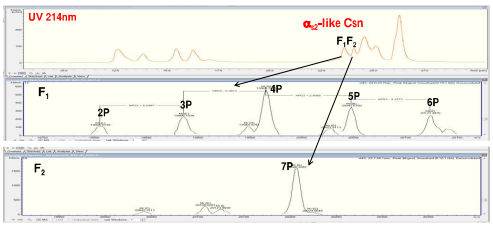
➢ Skim milk samples were analyzed by LC-MS. Protein separation was carried out on a reverse-phase column (RP-HPLC) using an increasing gradient of acetonitrile in water as previously described by Saadaoui *et al* (2014).

COMPARISON OF MILKS FROM THE WHITE POPULATION (PB) AND THE "SYNTHETIC" STRAIN (SS) on Day 10 of lactation (L10)

RP-HPLC (colonne C5 – 3µm -300A- 150x2.1 mm)



✓ **Similarity between the peak profiles among the two rabbit strains**
✓ **Different relative intensities (individual variability), especially among the milk samples of the SS strain**



CONCLUSIONS

✓LC-MS is a highly powerful method for a fine characterization of the main milk proteins (revealing post-translational modifications including phosphorylation isoforms)
✓ **Comparison of major milk proteins composition between rabbit PB and SS strains**:
- ➢ Qualitatively: very similar chromatographic profiles
- ➢ Quantitatively: no significant differences between the 2 groups
  higher variability in the SS group

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