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INNOVATIVE SEPARATION TECHNIQUES FOR THE EVALUATION OF THE QUALITY OF FOOD PRODUCTS

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1 INTRODUCTION

1.1 What does "Food Quality" mean?

Food quality can be considered both the most well-defined and the least well-defined concept in the food industry today. The difference depends upon who is defining it and the level at which it is measured.

If you were to ask any food scientist, e.g. nutritionist, food technologist, microbiologist, etc., 'What constitutes good quality in a food and how does one measure it?', the answer you get will be direct and unqualified. It will be an answer that is based on years of accumulated knowledge within the scientist's research discipline and will likely include a battery of standardized, instrumental tests to quantify food quality. Unfortunately, food scientists represent only a small percentage of people concerned with food quality. The remainder are consumers – those people whose definition of food quality drives the economy of the global food industry. Yet, it is precisely the consumer's definition of food quality about which we know the least and which we are most challenged to quantify. One of the major difficulties associated with the definition and measurement of food quality is that it is a relative concept. It is relative not only to who is doing the evaluation, but to a wide range of situational and contextual factors. However, this relativity is inherent in the term ***quality.*** Webster's dictionary defines quality as 'the degree of excellence which a thing possesses.' However, the word excellence is also a relative term, defined by Webster as 'surpassing goodness.' In turn, goodness is defined as 'better than average.' Obviously, there are no absolutes in the definition of the concept of quality; neither are there any absolutes in the concept of ***food*** quality. It is a concept that is relative to person, place and time. In spite of this fact, it is believed that food quality ***can*** be defined and that measures ***can*** be developed to quantify it [1].

All food products require analysis as a part of quality management program throughout the development process, through production and after a product is in market. The chemical

composition and physical properties of foods are used to determine the nutritional value, functional characteristic, and acceptability of the food products. The nature of the samples and the specific reason for the analysis commonly dictate the choice of analytical methods. Speed, precision, accuracy and ruggedness often are the key factors in this choice. Validation of the method for the specific food matrix being analyzed is necessary to ensure usefulness of the method. The success of any analytical method relies on the proper selection and preparation of food sample, carefully performing the analysis, and doing the appropriate calculation and interpretation of data [2].

1.2 Analytical methods: background

There is a continuing need for reliable analytical methods for use in determining compliance with national regulations as well as international requirements in all areas of food quality and safety. The reliability of a method is determined by some form of a validation procedure. The Codex Alimentarius Commission (CAC), for example, requires that in order for a method of analysis to be included in a Codex commodity standard, certain method performance information should be available. This includes specificity, accuracy, precision (repeatability, reproducibility), limit of detection, sensitivity, applicability and practicability, as appropriate. This very often requires an extensive collaborative study be undertaken to obtain the necessary data. Methods which have successfully undergone this performance review testing have been considered to be validated for purposes of analyses under Codex commodity standards.

The ideal validated method is one that has progressed fully through a collaborative study in accordance with international harmonized protocols for the design, conduct and interpretation of method performance studies. This usually requires a study design involving a minimum of 5 test materials, the participation of 8 laboratories reporting valid data, and most often includes blind replicates or split levels to assess within-laboratory repeatability parameters.

It is not practical or necessary to require that all analytical methods used for food control

purposes be assessed at the ideal level, especially methods for the determination of low-level contaminants in foods, such as veterinary drug and pesticide residues. Limiting factors for completing ideal multi-laboratory validation studies include high costs, lack of sufficient expert laboratories available and willing to participate in such studies, and overall time constraints [3].

1.3 Role and type of methods for Codex Alimentarius Commission

The Codex AlimThermal oxidationentarius Commission (CAC) has elaborated Principles for the Establishment of Codex Methods of Analysis, which classify methods of analysis as:

- Type I, Defining Methods;
- Type II, Reference Methods;
- Type III Alternative Approved Methods;
- Type IV, Tentative Methods.

This classification was designed primarily for the Codex commodity committees. Most methods currently listed for pesticide and veterinary drug residues would qualify according to this classification as tentative methods, while only those which had been subject to a collaborative study as defined by internationally accepted harmonized protocols would meet the requirements for Type I, II or III methods.

For Codex Type I, II or III methods, statistical parameter estimates for reproducibility are generally obtained from a collaborative study. A typical study of a determinative method conducted in accordance with the internationally harmonized ISO/IUPAC/AOAC protocol could require a minimum of up to five materials including blind replicates or split level samples, and eight participating laboratories [4].

A Level III method is defined as suitable for screening (usually semi-quantitative); a Level II

method is a determinative method that provides a quantitative estimate of the residue concentration; while a Level I method is a confirmatory method that unambiguously identifies the analyte and which may also provide quantitative information. Included with the intended use are recommended performance criteria for accuracy, precision and recovery.

1.4 Validation criteria for method performance

The general criteria for the establishment of method performance characteristics and subsequent selection of chemical analytical methods are given below. With one exception (limit of quantitation), these criteria are the same as those used by the CAC.

- ***Specificity*** - Details concerning specificity must relate at least to those substances which might be expected to give rise to an interfering signal when the measuring principle is used. In residue analysis, for example, they may include substances which give a response similar to the residue being measured. The details concerning specificity must quantitatively indicate the extent to which the method can distinguish between the analyte of interest and interfering substances under the experimental conditions. A check for random interferences should be performed by analysis of a set of representative blank samples.

- ***Accuracy*** - Accuracy refers to the closeness of agreement between the true value of the analyte concentration and the mean result that is obtained by applying the experimental procedure a large number of times to a set of homogeneous samples. It is closely related to systematic error and analyte recovery. The accuracy requirements of methods will vary depending upon the planned use of the results. Generally, accuracy at or below the maximum residue limit (MRL) or level of interest must be equal to or greater than the accuracy above the MRL or level of interest. The percent recovery of an analyte that is

added to a blank test sample is a related measurement that compares the amount found by analysis to the amount added to the sample. In interpreting recoveries, it is necessary to recognize that analyte added to a sample may not behave in the same manner as the same biologically incurred analyte (veterinary drug residues, for example). At relatively high concentrations, analytical recoveries are expected to approach 100%. At lower concentrations and particularly with methods involving a number of steps including extraction, isolation, purification and concentration, recoveries are often lower. Regardless of what average recoveries are observed, recovery with low variability is desirable.

- ***Precision*** - The precision of a method is the closeness of agreement between independent test results obtained from homogeneous test material analyzed under the stipulated conditions of use. Repeatability and reproducibility as defined in the Protocol for the Design, Conduct and Interpretation of Method Performance Studies [4] can best be estimated when the validation is carried out as a collaborative study. However, in the absence of a collaborative or other multilaboratory study, the laboratory must obtain an estimate of the method's repeatability and withinlaboratory reproducibility from data produced by that single laboratory. The precision of a method may also be estimated within http://www.google.ie/ a single laboratory using measurement reliability procedures as described in the ISO guide on the estimation of measurement uncertainty [5].

- ***Limit of quantitation*** - The limit of quantitation is the smallest measured content above which a determination of the analyte is possible with a specified degree of accuracy and repeatability (within-laboratory reproducibility). In general, the limit of quantitation of a method is associated with its limit of detection. In practice the limit of detection need only be determined when the limit of quantitation of the method approaches the limit specified in the applicable standard, such as a maximum residue limit. The limit of detection is generally

of lesser importance than the limit of quantitation because residue limits established by Codex, for example, are never zero.

- ***Sensitivity*** - This is the change in the analytical response divided by the corresponding change in the concentration of a standard (calibration) curve, i.e. the slope of the analytical calibration curve. A method is said to be sensitive if a small change in concentration of the analyte causes a large change in the analytical measurement. Although the analytical response may vary with the magnitude of the analyte concentration, it is usually constant over a reasonable range of concentrations [6]. In the ideal situation, the calibration curve becomes a straight line, expressing a direct linear relationship between analytical response and standard concentration.

- ***Practicability and applicability under normal conditions*** - This refers to the ease with which a method may be applied by those skilled in analysis. Preference should be given to methods of analysis which are applicable to a broad range of matrices and analytes. It also may include application to multi-residue methods. The method should be assessed over the relevant range of concentration, taking as a minimum half the value of the specified limit and twice the specified limit.

- ***Other criteria which may be selected as required*** - These may include ease of use, use of routine and versatile instrument*s*, availability of reagents, etc. (FAO Paper + references)

1.5 Analytical methods in brief: High Performance Liquid Chromatography

Chromatographic techniques showed an exorbitant development during this century. A Nobel prize was assigned to Martin and Synge in 1952 for their discoveries in this field. Nowadays these separation techniques have a great diffusion in chemical analysis of food products and in research of food adulterations. The use of chromatographic techniques is ubiquitarian and in several analytical cases indispensable thanks to their rapidity, efficiency and reliability. Moreover the possibility of coupling chromatographic with spectroscopic techniques, make them more interesting for researchers and conferring them more versatility.

Food products, thanks to their complex composition, formation of characteristic aroma compounds during technologies of production or bioactive compounds for consumer health, formation of marker compounds for adulterations, fraud or stage of conservation or metabolic compounds by microorganism with nutritional or technological concern, provide wide chance of use of chromatographic techniques for quali-quantitative evaluation of interesting compounds.

Chromatography is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases: a mobile phase is flowing through a stationary phase bed. The chromatographic process occurs as a result of repeated sorption/desorption steps during the movement of the analytes along the stationary phase. The separation is due to the differences in distribution coefficients of the individual analytes in the sample. Theoretical and practical aspects of LC have been covered in detail elsewhere [7-11].

In LC, the sample is injected by means of an injection port into the mobile-phase stream delivered by the high-pressure pump and transported through the column where the separation takes place. The separation is monitored with a flow-through detector.

1.6 Analytical methods in brief: Electrophoresis couples with Mass spectrometry

A core component of proteomics is the ability to systematically identify every protein expressed in a cell or tissue as well as to determine the salient properties of each protein (e.g. abundance, state of modification, involvement in multi-protein complexes, etc.). The technology for such analyses integrates separation science for the separation of proteins and peptides, analytical science for the identification and quantification of the analytes, and bioinformatics for data management and analysis. Its initial implementation consisted of the combination of high-resolution two-dimensional gel electrophoresis (2DE) for the separation, detection and quantification of individual proteins present in a complex sample with mass spectrometry and sequence database searching for the identification of the separated proteins [12].

To optimize separation, each dimension must separate proteins according to independent parameters. Otherwise proteins will be distributed across a diagonal rather than across the entire surface of the gel. Isoelectric focusing (IEF) and a discontinuous SDS gel system (Laemmly) were chosen because of the high resolution of each system and because they separate proteins according to different properties. Since the procedure is intended for analysis of total proteins, denaturation agents which solubilize most proteins are present during electrophoresis in both dimensions. This system permits simultaneous determination of molecular weights and approximate isoelectric points of proteins.

The instruments most commonly used for identification of protein of interest after 2DE separation can be grouped into two categories: single stage mass spectrometers and tandem MS-based systems. Single stage mass spectrometers, most notably the matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) instruments, were used in numerous projects for large scale protein identification by the peptide mass mapping technique. This method is particularly successful for the identification of proteins from species with smaller and completely sequenced genomes [13, 14]. Tandem MS instruments such as the triple quadrupole, ion-trap, and the recently introduced hybrid quadrupole-time-of-flight (Q-TOF) were routinely applied in LC-MS/MS or nanospray experiments

with electrospray ionization (ESI) to generate peptide fragment ion spectra suitable for protein identification by sequence database searching. The increased use of instrument control programs to automatically select specific peptide ions for collision-induced dissociation (CID) (data-dependent CID) was a notable trend with these MS/MS instruments.

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2 EXPERIMENTAL SECTION

The experimental section is divided into four chapters related to different topics which have been studied and deepened during this PhD project. These four chapters are also divided in two main categories according to the separation techniques used during the development of the experimental design. The scheme of this classification is indicated below:

High Performance Liquid Chromatography

Phenyl-lactic acid determination and application on production of salami;

Biogenic amines determination in low acidic salami;

Retention effect of oxidized polyphenols during analytical extraction.

Mono- and Two-dimensional Electrophoresis coupled with Mass Spectrometry

Study of the proteome of human milk and differences between milk for pre-term babies and term babies.

3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF PHENYLLACTIC ACID IN MRS BROTH

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3.1 Abstract

Phenyllactic acid (PLA) is an organic acid produced by some strains of lactic acid bacteria (LAB) and concentrations higher than 7.5 mg/ml inhibit growth of moulds and yeasts. Since PLA can be used to select LAB, a rapid, simple and cheap method for its determination is desirable. Typical methods for its analysis in broth are time-consuming, analytically complicated, and have poor recoveries. Herein we propose a simple and rapid method that does not require extraction, but only microfiltration of broth before injection in HPLC. The improved chromatographic conditions allow separation and quantification of PLA with a recovery of 98.7%. The method is highly reproducible with an intraday repeatability of the total peak area of 2.00%, while an interday repeatability of 2.69%.

Keywords: Phenyllactic acid determination; HPLC; Lactic Acid Bacteria (LAB).

3.2 Introduction

Production of fermentative compounds with antagonist action against moulds, yeasts or other microorganisms, is object of investigation for technologies of production of fermented foods. The capacity of lactic acid bacteria (LAB), to produce bacteriocin, compounds with inhibitory action against other bacteria or strains of the same species, has been known from long time [1-8]. *Lactobacillus plantarum* is a LAB of enormous technological relevance and is generally used as starter culture for production of several fermented foods [9-13]. Lavermicocca *et al.* [14] characterized a *Lactobacillus plantarum* strain that is able to produce both phenyllactic and 4-hydroxyphenyllactic acid, which are two organic acids able to inhibit the growth of moulds and yeasts. Phenyllactic and hydroxyphenyllactic acids have been also found as metabolites involved in formation of cheese flavor produced by LAB strains through degradation of phenylalanine and tyrosine, respectively [15-17]. However, PLA is the main responsable of inhibition activity and for this reason it is an effective marker of antifungal action of LAB. Production of this organic acid from LAB starter, or non-starter LAB in fermented foods, acquire significant technological importance due to their characteristic of antimicrobial action and impact on the formation of flavor-forming compounds. Therefore, a quantitative, simple, rapid and cheap determination of this organic acid from broth is required when its production could be used as a selection criteria for starter LAB.

Determination of PLA of previous works concerned especially analysis of rumen fluid [18-19]. In these works, PLA was determined as related product of metabolism of phenylalanine by rumen microorganism. An HPLC system with UV detection (215-220 nm) was employed for this determination and isocratic runs with methanol-50 mM sodium acetate buffer pH 6.5 (8:92, v/v) were performed after sample preparation. Most recent works looked at PLA as antifungal compounds produced by LAB. In these works, PLA determination requires liquid-liquid extraction with 4 · 30 ml of ethyl acetate after centrifugation and modification of pH to 2.0 with 10 M formic acid [20], or purification by solid-phase extraction (SPE) [21] after centrifugation and filtration of

bacterial supernatant. In both cases, quantification was carried out by HPLC/UV (210 nm) with water-acetonitrile gradient and run total time of 15 minutes for both methods. Herein we propose a simple and reliable method that does not require extraction or purification of bacterial supernatants for determination of PLA uisng HPLC with UV detection.

3.3 Experimental

3.3.1 Reagents and materials

The PLA-producing strain VLT01 of *Lactobacillus plantarum,* from the DIPROVAL collection (Bologna University), was grown on MRS liquid broth (Oxoid Ltd., Basingstoke, England). HPLC grade water, acetonitrile, and sulfuric acid (96%) were provided by Carlo Erba reagents (Milan, Italy). DL-3-phenyllactic acid (purity 97%) was purchased from Fluka (Buchs, Switzerland) and the SPE column (C18 EC) were obtained from Isolute, International Sorbent Technologies Ltd. (Hengoed, UK). Filters (0.45μm GMF w/GMF) were provided by Whatman (Middlesex, UK), while 0.22 μm type GS filters were purchased from Millipore (Billerica, MA, USA).

3.3.2 Bacterial supernatant preparation

Cells of the strain VLT01 of *Lactobacillus plantarum* harvested during the exponential growth phase was inoculated in 500 ml ($5 \cdot 10^5$ ufc/ml) of MRS broth and placed in a thermostat at 30° C under anaerobic conditions for 24 hours. At the end of incubation, samples were centrifuged at 4400 g for 10 min and subsequently filtered through a 0.22 μm filter. The solution was then divided in two aliquots to test two different sample preparations methods.

3.3.3 Sample preparation

Method 1

A C18 EC column was used for SPE purification. Column was activated with 20 ml of acetonitrile and equilibrated with 20 ml of HPLC grade water before sample loading. A washing step with 5% aqueous acetonitrile was followed by performing elution with 95% aqueous acetonitrile [18].

Chromatographic determination was carried out on washing extract (W) and elution extract (E) to evaluate possible repartition of phenyllactic acid in different fractions.

Method 2

Samples of bacterial supernatant were centrifuged and microfiltered using the same condition indicated in section 2.2 and directly injected in the HPLC system.

3.3.4 Chromatographic conditions

Analysis was carried out with a Waters (Milan, Italy) HPLC, equipped with a Waters 1525 binary pump, dual wavelength absorbance detector Water 2487 set at 210 nm, a Symmetry column C18 RP (150 x 4.6 mm, Waters, particle size 5 μm) at room temperature. Breeze 3.30 SPA software (Waters) was used for data acquisition and processing on a personal computer. The gradient used for elution is shown in Table 1. Phenyllactic acid was identified by comparison with retention time and co-elution of authentic standard solution.

Table 1
HPLC elution profile program

Time (min)	Acetonitrile (%)	Water (%)	Flow (ml/ min)
	25	75	1
3	25	75	1
4	50	50	1
6	50	50	1
8	100	0	1.3
12	100	0	1.3

3.3.5 Evaluation of recovery

Two ml of a standard solution of phenyllactic acid in MRS broth (4 mg/ml) in 200 ml with bacterial supernatant of VLT01 strain was prepared. Evaluation of recovery of phenyllactic acid was carried out through comparison of the spiked sample with the supernatant from the two different sample preparation methods.

3.3.6 Calibration and production curve

The quantification procedure was performed over the range of 5–500 μg/ml for above-mentioned sample using the peak area versus analyte concentration to make the calibration curves. The linear range was assessed using 7 different concentrations that were injected three times.

The curve of production was created monitoring PLA quantification during the exponential phase of microbial growth under the same conditions of the bacterial supernatant preparation indicated in section 3.1.2.2 without anaerobiosis. Samples were taken 8 hours after inoculation and every hour analyzed in duplicate during the successive 10 hours.

3.3.7 Statistical Analysis

The results reported are the average of three repetitions for sample analyzed with method 1, while microfiltered samples prepared using method 2 were injected 10 times on the same day (intraday precision, $n = 10$) and on two consecutive days (interday precision, $n = 20$). The relative standard deviations of the peaks area and migration times were determined.

3.4 Results and discussion

Some LAB produce proteic antifungal compounds [22-23], although these are formed during late inhibition due to cellular death with subsequent release of cellular material into the matrix through autolysis. In contrast, PLA is produced during the first hours of development reaching concentrations of 14 ppm within 8 hours of inoculation. Determination of PLA can represent an important selection criteria for LAB since they are utilized as a starter culture in fermented foods.

This organic acid is an effective marker of the ability of LAB to produce compounds with growth inhibitory activity [20]. Therefore it is important that a simple, effective, inexpensive and quantitative method is available for rapid screening on LAB to identify strains that possess enhanced inhibitory action.

Purification of bacterial supernatants in SPE according to the procedure by Ström *et al.* gave fair results, but only approximately 10% of the spike standard was recovered. We next focused our attention on the washing extract (W) carried out with 5% aqueous acetonitrile that was used in the purification step. Chromatographic analysis and evaluation of recovery of this fraction showed an effective repartition of PLA in both fractions, E and W, with a marked affinity of PLA to W fraction. For this reason it was appropriate to verify the performance of SPE. In fact, depending on the batch, activation, quality of extraction solvents and environmental condition, SPE columns can provide very different results. Using of a spiked standard, as reported in Table 2, recovery was completely unsatisfactory.

Table 2

		PLA (mg/l) ± SD	RSD (%)	Recovery (%)
SPE method	E	12.4 ± 1.1	8.9	10.5
	E + Std (40.3 mg/l)	16.6 ± 1.4	8.6	
	W	23.0 ± 0.3	1.2	63.2
	W + Std (40.3 mg/l)	48.3 ± 0.9	1.9	
Filtration method	Fil	43.3 ± 0.9	2.0	98.7
	Fil + Std (40.3 mg/l)	83.1 ± 0.5	0.5	

Evaluation of recovery of standard addition for SPE and Filtration method; PLA amounts are the average values of $n = 3$ repetitions for SPE method and $n = 10$ repetitions for Filtration method
W = Washing extract; E = Elution extract; Fil = Filtered sample
SD= standard deviation; RSD = relative standard deviation

In our opinion it was also possible that interactions between chemically complex bacterial supernatants and the stationary phase of column make elution of PLA difficult and a large proportion of it was retained in the column. Alternatively, the method was set up with small concentrations of PLA, and that high concentrations of PLA overloaded the column.

Subsequent trials were carried out on microfiltered samples. Bacterial supernatant was centrifuged

to remove residual bacterial cells and filtered with 0.22 μm filter before injection. Trials carried out on these samples gave encouraging results. PLA in these samples eluted in a dirty zone of the chromatogram making quantification unreliable. An improved method was proposed to elute all interfering compounds in the first minutes of the chromatographic run to obtain clean chromatogram in PLA elution area (Fig. 1).

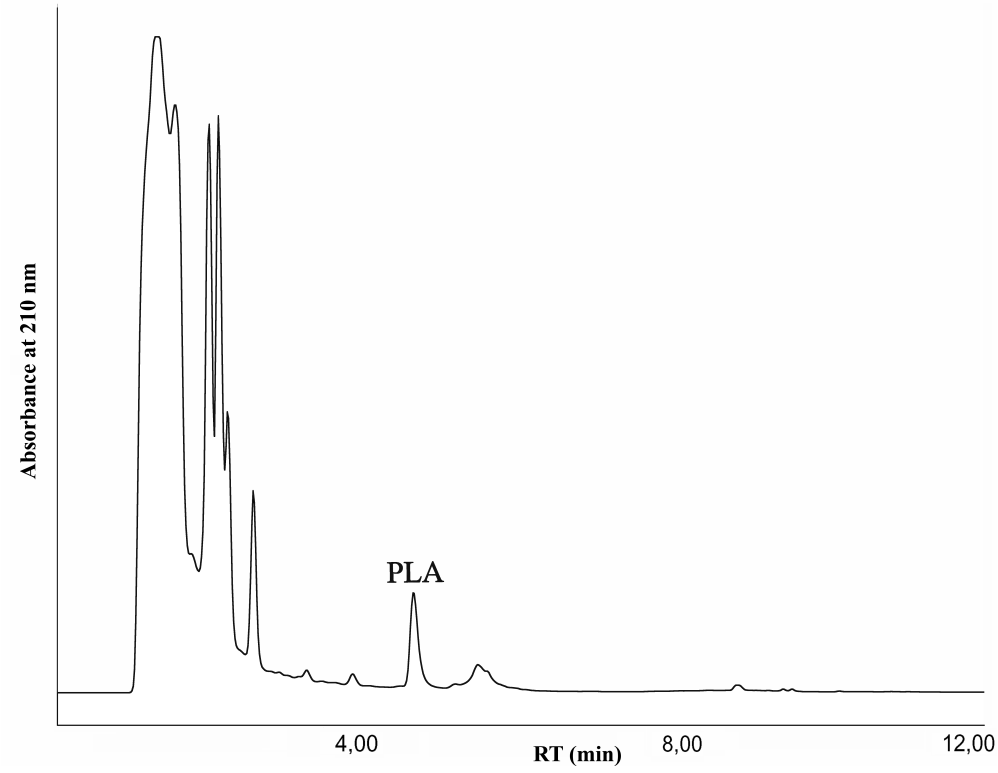

Figure 1: HPLC trace of a microfiltered samples with the modified gradient.

A modified gradient indicated in Table 1 showed excellent reproducibility and the new method of samples preparation (method 2) allows quantitative recoveries (Table 2). This new method was used for subsequent evaluations of efficiency.

3.4.1 Validation of the HPLC method

Intraday repeatability (expressed as R.S.D.) of the migration times was 0.38% whereas the interday repeatability was 1.21%. Intraday repeatability (expressed as R.S.D.) of the total peak area was 2.00%, while the interday repeatability was 2.69%. As expected, the intraday precision was greater

than interday precision and the method demonstrated good overall reproducibility. The sensitivity of the method was assessed on microfiltered samples in a solution of 1 µg/ml, which gave a signal-to-noise ratio of approximately 3, corresponding to the limits of detection of the method. The quantification procedure was performed as indicated in section 2.6. The results of linear regression were as follows ($A = mc \pm q$, where A is the peak area, c is the analyte concentration expressed as µg/ml, q is the y-intercept and r^2 is the correlation coefficient): $A = 45715c + 227429$ ($r^2 = 0.997$).

The efficacy of the method was also tested with a curve of production. This resulting curve is shown in figure 2 together with the calibration curve.

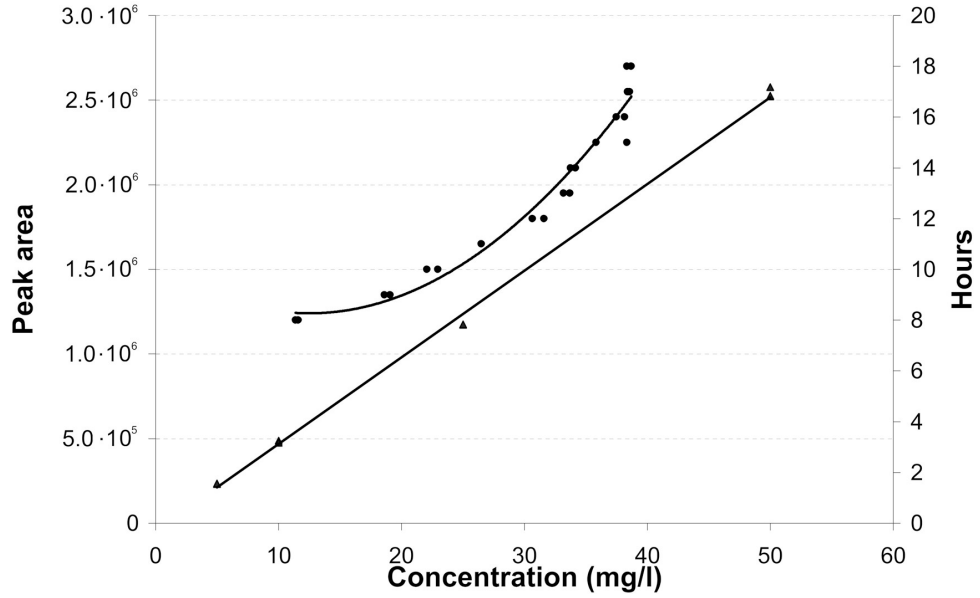

Figure 2: PLA quantitative evaluation:
▲ Calibration curve of PLA versus peak area;
● Production curve of PLA versus hours after inoculation.

3.5 Conclusions

Compared with previous determinations this new method provides significant improvements for determination of PLA:

1. Quantitative recoveries of about 99%;
2. Strong reproducibility and reliability as showed in section 3.1;
3. Rapidity: simple chromatographic run versus 2 hours of SPE purification or 1 hour of

liquid-liquid extraction;

4. Limits in consumption of solvents: only solvents employed for HPLC gradient versus a liquid-liquid extraction with 120 ml of ethyl acetate for sample or an SPE purification with about 100 ml of water and 100 ml of acetonitrile for sample before HPLC injection;

For all this reason the improved method described herein results efficient, reliable and reproducible and also provides quantitative recoveries, methodological simplicity and a notable contribution to respect of lab operator and environment.

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4 ANTIFUNGAL ACTIVITY OF LACTOBACILLI ISOLATED FROM SALAMI

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Keywords: Lactobacilli, moulds, starter, phenyllactic acid, hydroxy – phenyllactic acid, antagonism

4.1 Abstract

Sixty-five strains of lactobacilli isolated from salami were tested for their antifungal activity in early and late phase of growth.

Ten strains showed inhibitory activity in early phase of growth toward moulds, as *Aspergillus* and *Penicillium.* The active compounds identified were phenyl – lactate and hydroxy-phenyl-lactate.

All strains tested had activity in the late phase, after autolysis. The compounds released was peptidic and showed antifungal activity.

4.2 Introduction

It's well know that some lactic acid bacteria produce bacteriocins, that inhibit the growth of the same and other species of bacteria [1-2].

In the past was also demonstrated the action of lactic acid bacteria towards moulds: *Lactobacillus casei* subsp. *rhamnosus* produces a small molecular weight molecule that can inhibit the growth of *Aspergillus parasiticus* [3].

Following this report, several studies showed the peptidic nature of the antifungal compounds produced by *Lactobacillus acidophilus* [4], *Lactobacillus coryniformis* subsp. *coryniformis* [5] and *Lactobacillus plantarum* [6]. Moreover in addition to peptides, other authors [7-8] demonstrated that a mixture of short-chain fatty acids is produced by *Lactobacillus sanfranciscensis*. *Lactobacillus plantarum* synthesizes a number of different substances including benzoic acid, methylhydantoin, and mevalonolactone, which have additive antifungal activity [9]. Recent studies [10] confirm that the inhibitory activity of *Lactobacillus plantarum* can be attributed to the organic acids phenyl-lactate and 4-hydroxy-phenyl-lactate.

Moulds play an important, but not univocal, role in ripening of fermented food by lactic acid bacteria. Their role is generally considered positive in the curing and aging of salami when they develop on the surface, in fact:

- regulate of the flow of water from the inside towards the outside;
- brings the pH to a value of 5.6-5.7 with a deacidification;
- contribute to the formation of aroma and taste trough lipolysis [11] and proteolysis [12].

The role of moulds in cheeses is very important for particular products as Roquefort, Shilton, Camembert and Gorgonzola. In other cheeses the role may be considered negative because their growth on the surface cause alteration, with production of mycotoxins and harm the external aspect of product [13-14].

Regarding these considerations, the present study was carried out with the aim of understanding if

the inhibitory activity towards mould can be considered character for the selection of the lactic acid bacteria used as starter cultures for salami and the inhibition is due to compounds that are formed during the fermentative or post-fermentative phases. For this purpose, we examined the most frequently observed species in salami cured naturally, namely *Lactobacillus plantarum* and *L. sakei* [15-17] and the moulds that are often found on the sausages and cheeses or employed as starter [14, 18].

4.3 Material and methods

4.3.1 Microorganisms

A total of 65 strains of *Lactobacillus* isolated from salami from different origins were used, which are part of the collection at DIPROVAL; these strains are indicated with the letters VLT followed by the registration number. Some type strains from DSMZ collection were also used, including *L. plantarum* DSMZ 20174^{T} and *L. brevis* DSMZ 20054^{T}.

Phenotypical characterization of *Lactobacillus* strains was carried out whit API 50 CH System (Bio-Merieux).

The inhibitory activity of *Lactobacillus* species was compared to several species of mould that are often found in association with lactic acid bacteria in fermented foods.. Strains of these species were from our collection and DSMZ collection.

Lactic acid bacteria were stored at 4 °C fixed in MRS Agar (OXOID) and subcultured monthly; moulds were stored in Sabouraud agar (OXOID) and were also subcultured monthly.

4.3.2 Determination of inhibitory activity

Inhibitory activity of compounds produced during the development phase was determined in plates using the overlay technique [5]. Lactic acid bacteria were plated on the surface in 10 mm lines on 90 mm plates containing 25 ml MRS agar. Plates were incubated in anaerobiosis at 30 °C for 48 hours. After growth of lactic acid bacteria, 10 ml of Sabouraud were added (agar 7 g L^{-1}), which had

been previously inoculated with 10^5 CFU mL^{-1} of conidia. Plates were then incubated in aerobic conditions at 25 °C and after the growth of fungi were evaluated for inhibition halos around the areas of growth of lactic acid bacteria. The inhibitory capacity was scored as follows: - no inhibition, + inhibition halo up to 8 mm from the plating line, ++ halo between 9 and 15 mm; +++ halo larger than 15 mm.

The inhibitory activity of compounds produced during the post-fermentative phase was determined using a modified version of the protocol previously described [19], which foresees the use of the plate diffusion method described for the determination of the bacteriocine [20]. In particular, lactic acid bacteria were allowed to grow at 30 °C and then left alone for 30 days to favor autolysis, confirmed by observation with a scanning electron microscope (SEM). The growth medium was recovered, centrifuged (5000 rpm for 10 min) to eliminate cells and filtered (0.20 µ m; Albet Jacs). The supernatants were concentrated by lyophilization and resuspended in phosphate buffer (50 mM, pH 7.0) to 15 times the original concentration. Of this, 125 µ l was placed in 9 mm wells obtained in 90 mm plates containing Sabouraud (agar 7 g L^{-1}), which had been previously seeded with *Penicillium nalgiovense* that is sensitive to inhibitory activity [19].

4.3.3 Determination of the physico – chemical characteristics of the inhibitory substances

The inhibitory capacity of the species harboring the greatest activity was characterized in both early and late phases. In the latter case, the activity was compared with a species that had no early inhibitory activity. Mixtures of compounds produced in both early and late phases were characterized, obtained by fermentation for 48 hours for both the early and late phase after 30 days. In both cases, cultures were allowed to develop in 250 mL MRS in 500 mL Erlenmeyer flasks at 30 °C. The supernatant was collected by centrifugation (5000 rpm, 5 min), sterilized by filtration through 0.20 µm filters, concentrated by lyophilization, and resuspended in phosphate buffer (50 mM, pH 7.0) at 15 times less the original volume. The inhibitory activity of the concentrate was evaluated using 9 mm wells as previously described, both before and after the steps recommended

by various authors [5, 10].

In particular:

- to determine if the compound had a peptidic structure, the concentrated, buffered supernatant (pH 7.0 with 4N NaOH) was subjected to digestion with the following enzymes: proteinase-K (Sigma), trypsin (Sigma), and protease (Sigma) at 37 °C for 1 hour. After the reaction, the concentrate was brought to pH 3.5 with HCl.
- heat resistance was determined by subjecting the supernatant to different thermal treatments: 80 and 100 °C for 10 min and 60 min.
- the influence of pH was examined by assessing the inhibitory activity of the concentrate at different pH values: 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 7.0.

4.3.4 Determination of phenyl-lactic acid and hydroxy-phenyl-lactic acid by HPLC

The concentration of phenyl-lactic and hydroxy-phenyl-lactic acid in supernatants was determined, as reported [21], with Waters (Milano, Italy) HPLC equipment using a Symmetry column C18 RP (150 x 4.6 mm), Waters (Milano, Italy), particle size 5 μm, kept at room temperature.

4.3.5 Image acquisition

Measurement of the inhibition halos was performed after digital acquisition of the plate images using a Bio-Rad Gel-Doc 2000 and elaborated using Adobe Photoshop 6.0. Scanning Electron Micrographs (SEM) were taken with a Hitachi 510 S. Samples were prepared using the method described by Bottazzi & Bianchi [22].

4.4 Results and discussion

4.4.1 Spectrum of inhibitory activity during the fermentative phase

Sixty-five strains of *Lactobacillus* were tested for their antifungal capacity during the growth phase using the double overlay technique with *Aspergillus candidus* DSMZ 814^{T} and *Penicillium nalgiovense* MF BP3 as the sensitive strains. As shown in Table 1, 54 of the 65 strains had no

inhibitory activity.

Table 1: Inhibitory activity of 65 strains of *Lactobacillus* vs. *Aspergillus candidus* and *Penicillium nalgiovense* strains

Species	Strains examined	*Aspergillus candidus* DSM 814^T	*Penicillium nalgiovense* MF BP3
L. plantarum	DSMZ 20174, VLT02, VLT04, VLT31, VLT33, VLT34, VLT35, VLT36, VLT37, VLT38, VLT39, VLT62, VLT71, VLT72, VLT78, VLT 154, VLT 156, VLT154, VLT157, VLT158, VLT160, VLT302, VLT310, VLT452, VLT454, VLT456, VLT457, VLT458, VLT1510, VLT4510,	-	-
L. plantarum	VLT01	+++	+++
L. plantarum	VLT73	+	+
L. plantarum	VLT301	+	++
L .plantarum	VLT304	+	++
L .plantarum	VLT307	++	+
L .plantarum	VLT451	++	+
L .plantarum	VLT452	++	+
L. sakei	VLT32 , VLT74, VLT96, VLT130, VLT148, VLT 159, VLT160, VLT710	-	-
L. pentosus	VLT75, VLT76, VLT77, VLT308, VLT309, VLT310, VLT459	-	-
L. pentosus	VLT308	++	+
L. pentosus	VLT310	++	+
L. pentosus	VLT459	++	+
L. curvatus	VLT152, VLT306, VLT96, VLT166	-	-
L. mali	VLT03, VLT154	-	-
L. mali	VLT112	+	+
L. brevis	DSMZ 20054, VLT118, VLT166	-	-

Nine strains had marked inhibitory capacity, particularly intense in VLT01, and produced distinct halos. Two strains had weak inhibitory activity. The typical aspect of the inhibition halos is shown in Figure 1.

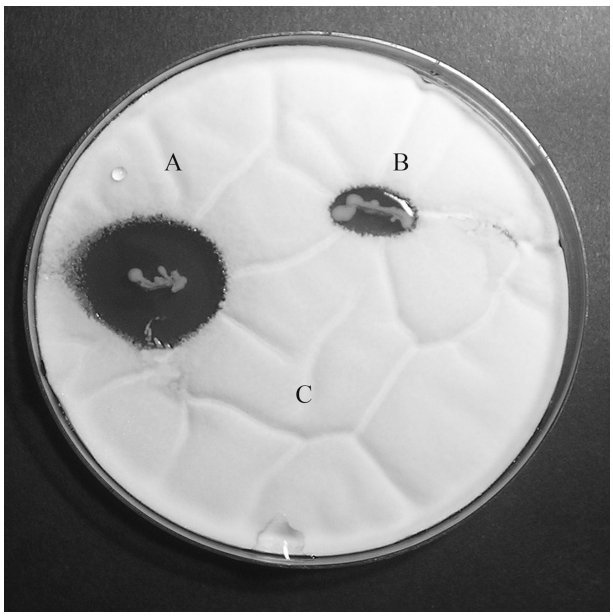

Fig. 1. Plate obtained using the overlay technique. (a) Strong inhibition by VLT01; (b) weak inhibition by VLT304; (c) no inhibition by VLT32.

The most active strain, *Lactobacillus plantarum* VLT01, was chosen for further screening on the sensitivity of various mould species. VLT 73 and VLT 304 were also used as they possessed intermediate activity and VLT32 as negative control. As shown in Table 2, VLT01, VLT73 and VLT304 exhibited activity against all the moulds employed, in particular against *Aspergillus* (*A. flavus, A. ochraceus* and *A. candidus*) producing the most dangerous micotoxins and against *Penicillium* that often colonize salami casings and cheese crusts, as *P. verrucosum* var. *cyclopium, P. nalgiovense, P. camemberti* and *P. roqueforti*. VLT32 did not show any inhibitory activity as expected.

Table 2: Inhibitory activity of 4 strains representative of *Lactobacillus* on various species of mould.

Mould	Specie	Strains with action: strong VLT01	medium VLT304	medium VLT73	absent VLT32
DSMZ 814	*Aspergillus candidus*	+++	+	+	-
DSMZ 1240	*Geotrichum candidum*	+++	+++	+++	-
DSMZ 1959	*Aspergillus flavus*	++	+	++	-
MFBP3	*Penicillium nalgiovense*	+++	++	+	-
MF4	*Aspergillus ochraceus*	+++	+	+	-
MF5	*Penicillium camemberti*	+++	++	+++	-
MF11	*Moniliella* spp.	+++	+++	+++	-
MF12	*Aspergillus fumigatus.*	++	+	++	-
MF40	*Mucor racemosus*	+	+	+	-
MF80	*Penicillium nalgiovense*	+++	+	++	-
MF117	*Wallemia sebi*	+++	+++	+++	-
MF123	*Penicillium verrucosum*	++	+	+	-
MF128	*Eurotium herbariorum*	+++	+++	+++	-
MF139	*Penicillium chrysogenum*	++	+	++	-

4.4.2 Inhibiting activity during the late phase

The inhibiting activity during late phases was determined by plating 30-day-old supernatant concentrates in wells using the four strains mentioned previously, which were found to have different inhibiting activities during the early phase. From the results in Table 3, it is evident that even one strains that do not have inhibiting activity in the early phase, while in the late phase after autolysis inhibit the growth of mould near the wells. This confirms previous studies [19], demonstrating that inhibitory activity is due to compounds that are released following autolysis.

Table 3: Inhibitory activity of selected strains in the late phase after autolysis.

	Aspergillus candidus		*Penicillium nalgiovense*	
After:	2 days	30 days	2 days	30 days
Lactobacillus plantarum VLT01	+++	+++	+++	+++
Lactobacillus plantarum VLT304	+	++	++	+++
Lactobacillus plantarum VLT73	+	++	+	++
Lactobacillus sakei VLT32	-	++	-	++

4.4.3 Physico - chemical characteristics of compounds produced in the early phase

The supernatant concentrate from the early phase treated with proteolytic enzymes showed no differences in inhibitory capacity with respect to untreated supernatant, which would exclude that the compound has a peptidic nature. Similarly, thermal treatment had no effect on inhibition of growth. The compound was however sensitive to changes in pH and concentrated supernatant showed marked antifungal activity at acidic pH that was reduced at neutral pH (Table 4).

Table 4: Physico - chemical characteristics of compounds produced in the early phase by *L. plantarum* VLT01

Treatment	Activity (%)
Concentrate 15 fold	100
pH:	
3.5	100
4.0	64
4.5	45
5.0	36
6.0	6
7.0	0
Proteolitic enzymes	
Trypsin	98
Protease	98
Proteinase K	99
Heat treatment	
80 °C x 10 min	100
100 °C x 10 min	98
80 °C x 60 min	98
100 °C x 60 min	98

HPLC analysis indicated, for the VLT01 strain, a phenyl-lactate and hydroxy- phenyl-lactate concentration of 46.6 and 67.6 mg L^{-1} respectively. For VLT 32 are both not detectable.

The values obtained are in agreement with other authors [10, 21, 23] in that phenyl-lactate was implicated in the inhibitory activity in the different species of *Lactobacillus*.

4.4.4 Physico - chemical characteristics of the compound produced during the post-fermentative phase

The supernatants of lactic acid bacteria cultures were studied after aging for 30 days, which is a sufficient time for autolysis to occur. In strain VLT01, which is active at an early stage, the inhibitory activity was maintained, but showed significant differences with respect to those previously seen. In particular, the activity was less sensitive to variations in pH with respect to the early phase and the products were found to be sensitive to enzimatic treatment. The tests carried out on aged supernatants were sensitive only to the action of proteinase K that brought about significant changes in the intensity and diameter of the relative inhibition halos.

In the case of thermal treatment (80 °C for 60 min) a residual activity was found similar to that seen in the early phase that was dependent on pH and thus, likely due to phenyl lactate. This results were confirmed by the presence of 7.54 mg L^{-1} of phenyl-lactate. In the case of strain VLT32, the inhibitory activity was completely sensitive to heat; in fact, treatment at 80 °C for 60 min was sufficient to completely inactivate the compound (Fig. 2).

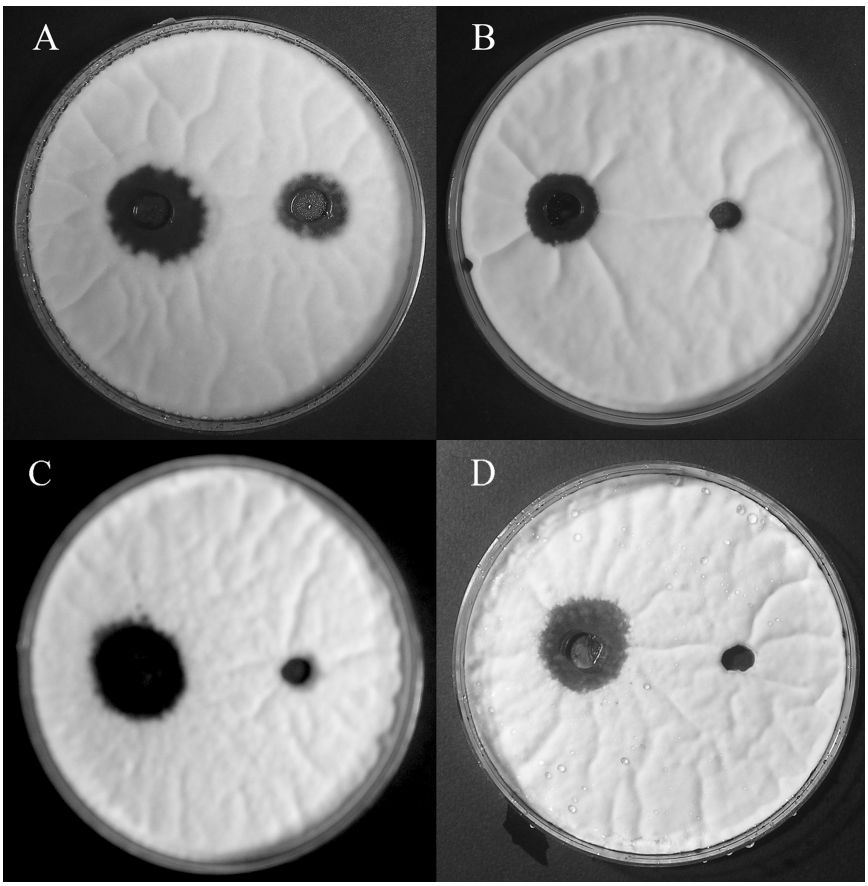

Fig. 2. Plates obtained with supernatants of strains aged for 30 days and thermally treated (80° for 60 min). (a) Strain VLT01, pH 3.5; (b) strain VLT01, pH 7.0; (c) strain VLT32, pH 3.5; (d) strain VLT32, pH 7.0. For each plate, the left wells were untreated, while the right wells were heat-inactivated.

The former data would confirm that the compound released was peptidic as already suggested by previously studies [19], which demonstrated that autolysis is responsible for the release of active biological compounds that are not apparent in the early phase. In fact, VLT32 was not active in the early growth phases, but produces a supernatant that is capable of inhibition after ageing.

4.5 Conclusions

The inhibitory activity of lactobacilli against moulds is double. This action can be realized in different times and caused by different factors. The first one, which is realized during fermentation and is due to the formation of compounds, as phenyllactic acid, and it is a characteristic of some strain. The second action, realized at the end of cell growth, is due to the release of peptidic compounds. Respect to the first one, it is a common characteristic of all the strain as the physiological consequence of cellular autolysis.

The inhibitory activity in the fermentative phase, is presented in some strains of *Lactobacillus,* has interesting technological possibilities for a variety of fermented food products, as dry fermented sausages or cheese. In fact, this lactic acid bacteria is often used as a starter to guide fermentation, and its behavior towards moulds must be considered as one of the main selection characteristic. The choice of the strain to use in this regard for fermentation is naturally based on the desired results.

Post fermentative activity is an important phase especially during production of salami, and it acts in the late phase with an antagonist action versus toxygen aspergillis but also versus *Penicillium* moulds before their growth become excessive, influencing in a negative way the quality of the products.

Acknowledgments

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5 COMBINED USE OF STARTER CULTURES AND PRESERVATIVES TO CONTROL PRODUCTION OF BIOGENIC AMINES AND IMPROVE SENSORIAL PROFILE IN LOW ACID SALAMI

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5.1 Abstract

The combined effect of starter culture, nitrites and nitrates has been studied in low acidity salamis, typical products of Northern Italy. Nine batches have been prepared, combining three different inoculations of starter cultures (control, *Lactobacillus plantarum* and *Lactobacillus plantarum* together with *Kocuria varians*) with three different preservatives (control, sodium nitrate and sodium nitrite). All the batches showed a good fermentation process with a proper pH decrease and which was quicker in batches inoculated with *L. plantarum*. The use of starter cultures and in particular the use of nitrites allowed the control of the proliferation of *Enterobacteriaceae* and enterococci. The accumulation of biogenic amines, especially putrescine, cadaverine, tryptamine and tyramine, in salami ready for consumption (sixty days ripening) was strongly affected by the presence of *Enterobacteriaceae* and enterococci.

Results obtained showed that the combined use of adequate preservatives and starter cultures allows the production of safer products with improved sensorial profile.

Keywords: salami, biogenic amines, nitrate, nitrite, starter cultures, sensorial profile.

5.2 Introduction

The production of salami in Italy is achieving noticeable importance, due to the interest of customers in typical products, guaranteed through Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) acknowledgment (Reg. CE 510/06). Salami consumption has been criticized by nutritionists describing salami as a food rich in fat and salt, as well as in potentially toxic compounds such as biogenic amines.

Biogenic amines (BA) are organic bases with aliphatic (putrescine and cadaverine), aromatic (tyramine and phenyl-ethylamine), or heterocyclic (histamine and tryptamine) structure. They can be found in several foods and are mainly produced through microbial decarboxylation of aminoacids [1]. While BA in low concentrations are essential for many physiologic functions, in high concentration they can have deleterious effects [2]. For instance, histamine and tyramine are particularly involved in the “histaminic intoxication”, normally known as poisoning from scombroid fish [3] and in the “cheese reaction” [2]. Excessive oral consumption of BA causes headaches, hypo- and hyper-tension, nausea, cardiac palpitation, renal intoxication, serious cases of cerebral haemorrhage and even death [4]. BA are normal constituents of food products such as cheese, wine, beer, sauerkraut, fish products and fermented meat as the result of enzymatic degradations or fermentative processes [1]. Accumulation of BA in food requires the availability of reaction precursors (aminoacids), presence of microorganisms having aminoacid-decarboxylase as well as adequate conditions that allow microbial growth and activity [5].

Recently, several authors have discussed the problem of presence of BA in fermented salami and have set up analytical methods for their determination and quantification. In fermented salami, BA originate from presence of aminoacids, accumulated during the ripening phase as a result of

proteolytic activity, as well as several microbial groups with decarboxylasic activity: *Pseudomonas*, *Enterobacteriaceae*, *Enterococcus* and *Lactobacillus* [6-7].

Many authors [8-10] suggest the use of selected starter cultures to control the level of BA in salami, while other authors have indicated that sugar can also be used [11-12] Gonzalez-Fernandez et al. [12] suggest the simultaneous use of both methods.

The use of nitrite alongside the use of selected starter cultures, which limits the growth of *Enterobacteriaceae,* has been suggested in traditional Spanish [9] and Turkish [13] sausages as a method to control the amount of biogenic amines.

Typical Italian salami, such as Felino, are characterised by features that can promote the accumulation of BA: high pH if compared to other northern European salami products, low sugar levels and prolonged ripening [7].

Based on these considerations, this work evaluates the role of adding starter culture and preservatives to control the accumulation of BA during the production of Felino Style Salami with low sugar content. For this purpose, a starter culture of lactic acid bacteria (LAB) has been selected and used for the production of salami with the addition of nitrites or nitrates.

5.3 Materials and methods

5.3.1 Selection of lactic acid bacteria

Sixty-five strains of *Lactobacillus* from the "Dipartimento di Scienze degli Alimenti Collection" (University of Bologna) were tested for their fermentative strength according to Buckenhuskes [14] and for the absence of decarboxylasic activity in MRS broth, modified according to Bover-Cid and Holzapfel [15]. Biogenic amines in the broth were analysed as reported below.

Strains without decaboxylasic activity were tested to analyse fermentative activity in meat [16] in order to choose the strain with highest acidogenic power and the best influence on sensorial characteristics. For this purpose, overnight cultures of each strain grown at 30°C in MRS broth (Oxoid, Basingstoke, UK) were centrifuged (17000 *g* for 15 min, Centrifuge 5415 R, Eppendorf,

Hamburg, Germany), washed twice in 0.9% (w : v) NaCl and re-suspended in the same solution. Each strain was then inoculated in minced pork loin (10^7 CFU/g) and incubated at 25°C in sterile bags under vacuum. After 48 h incubation, pH and absence of unpleasant smell were evaluated as described below.

5.3.2 Formulation of starter culture and sausages manufacture

The LAB strain selected as described above were inoculated in MRS broth (Oxoid), and incubated at 30°C for 24h under anaerobic conditions. After centrifugation and rinsing steps using 0.9% (w:v) NaCl, each strain was frozen (-18°C) in the same solution until its use in the manufacture of salami. *Kocuria varians* MIAL 12, previously selected for its technological properties [17] was maintained on slant of Mannitol Salt Agar (MSA, Oxoid) at 4°C until use. This strain were inoculated in the same liquid media at 30°C for 48h and treated as reported for the LAB strain.

Before the use, microorganism was revitalised for 16 h at 30°C in MRS broth (OXOID) for the LAB and Mannitol Salt Broth (OXOID) without phenol red for *Kocuria* strain.

Sausages were produced by using 73% lean pork, 27%, pork fat, NaCl (23 g/kg) and glucose (2 g/kg), without spices. After chopping and mixing, the mixture was divided into nine batches; starter cultures and preservatives were added to each batch according to the distribution shown in Table 1.

Table 1: Experimental design. Letters indicates the batches obtained

starter cultures	Preservative		
	$NaNO_3$ (250 mg/kg)	$NaNO_2$ (150 mg/kg)	None
Lactobacillus plantarum VLT 73 (10^6 cfu/g) *Kocuria varians* MIAL 12 (10^5 cfu/g)	A	B	C
Lactobacillus plantarum VLT 73 (10^6 cfu/g)	D	E	F
None	G	H	I

Batches A-C were inoculated with a starter culture composed of *L. plantarum* VLT73 and *K.*

varians MIAL12 prepared as reported above while batches D-F were inoculated with *L. plantarum* VLT73 alone. Batches G-I were not inoculated with starter cultures to serve as controls. Moreover, sodium nitrate (250 mg/kg) was also added to batches A, D, G whereas sodium nitrite (150 mg/kg) was added in batches B, E. Mixtures were stuffed into natural casings.

Twelve sausages of approximately 1000 g were produced for each batch. Sausages were placed in a drying chamber at 23°C and 90% of relative humidity (RH) for 48 h. Thereafter, sausages were held in the ripening chamber at 13°C and 80–70% RH for further 58 days.

5.3.3 Microbiological and physicochemical analyses

Microbiological analyses were performed at time zero (meat mixture prior to stuffing), and after 3, 10, 24, 45 and 60 days of ripening. For this purpose, 20 g of sausage (without casing) were removed under aseptic conditions and homogenized for 2 min with 180 ml of 0.9% (w : v) NaCl using a Stomacher (Lab Blender Seward, London, UK). The solution was then used in order to prepare decimal dilutions.

Enterobacteriaceae were counted on VRBGA (Oxoid) incubated for 24 h at 37°C; Enterococci on Slanetz and Bartley Medium (Oxoid) after 48 h at 45°C. Lactic acid bacteria (LAB) were cultured on MRS agar (Oxoid) at 30°C for 96 h under anaerobic conditions. Gram positive - coagulase negative cocci were cultured on MSA (Oxoid) at 30° for 72 h. Three replicates were carried out for each microbial count.

A pH-meter (ORION) equipped with a penetration probe electrode (Orion) was used to measure acidity directly in sausages.

5.3.4 Detection of biogenic amines

Biogenic amines were determined at time zero and after 60 days of ripening according to Hwang et al., [18]. Ten grams of salami from each batch were minced and homogenised after the addition of 20 mL of a 5% aqueous solution of trichloroacetic-acid using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). Homogenised samples were then placed in thermostat at 75°C for 30 min and

subsequently centrifuged (Beckman Centrifuge J2-21, Palo Alto, CA - USA) at 5000 *g* for 10 min. The supernatant was filtered on filter paper Whatman n° 40 (Maidstone, UK). The extraction procedure was repeated on the pellet and the acid extracts were combined and made up to a volume of 50 mL using the extraction solvent. One mL of NaOH 2N and 20 µL of benzoyl-chloride were added to 2 mL of extract (or standard mix). After mixing the sample were derivatised incubating it in a water bath at 30°C for 40 minutes. After this step, 2 mL of a urea saturated solution were added to samples. Tubes were then mixed for 30 seconds by vortex and placed again in a water bath at 30°C for 10 minutes. In order to extract the derivatised compounds to the organic phase samples were treated with 3 ml of diethyl-ether and mixed for 1 minute by vortex. Phase separation was carried out by centrifugation (3000 *g* for 5 minutes) and the organic phase was dried using a slow flow of nitrogen. The dried extract was solubilised with 2 mL of a *n*-hexane/*iso*-propanol (4:1) solution and filtered using a 0.20 µm filter for the subsequent chromatographic analysis. Analysis was carried out with a Waters HPLC (Milan, Italy), equipped with a Waters 1525 binary pump, dual wavelength absorbance detector Water 2487 set at 250 nm, symmetry C18 column. Solvent A was HPLC grade water (Carlo Erba reagents) and solvent B was HPLC grade methanol (Carlo Erba reagents). An elution gradient was programmed for solvent B as follows: 50% for 0.5 min, from 50% to 15% in 6,5 min followed by 5 min at 15% then from 15% to 50% methanol in 2 min, followed by 2 min in 50% methanol. A flow rate of 0.8 mL/min was employed and 20 µ L of sample were injected. Breeze 3.30 SPA software (Waters) was used for data acquisition and processing on a personal computer. All the biogenic amines were tentatively identified by comparison of retention time and co-elution with the commercial standard compounds (Sigma, St. Louis, MO, USA).

5.3.5 Biogenic amine quantification

Calibration curves were performed over the range of 5–500 µg/mL for each amine standard solution using the peak area versus analyte concentration in order to quantify the BA. The linear range was

assessed using seven different concentrations that were injected three times.

5.3.6 Sensory evaluation

A panel of 9 assessors (TINVAL) previously trained in descriptive analysis for meat products according to Chiavari et al. [19] were used for the sensory evaluation

Smell and off-flavour of minced pork loin were evaluated to select strain suitable to produce sausages.

Sausages were evaluated at the end of the ripening, i.e. after 60 days, considering appearance, smell, aroma and texture. Features of the salami were appreciated by observation, light manipulation, and/or taste. The parameters are listed in the order in which they were evaluated during the test. Intensity was marked on an arbitrary scale with points from 1 to 7.

5.3.7 Statistical analysis.

Data regarding sensory analysis and determination of biogenic amines were statistically analysed using the ANOVA procedure. Pearson correlation was used to find significant relation between bacterial counts and level of biogenic amines. The analysis of data was carried out by using the statistical package SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

5.4 Results and discussions

5.4.1 Lactobacillus starter selection.

Out of 65 analysed strains, 25 showed a good fermentative strength and high acidogenic power, characteristics which suggested their use for the fermentation process. For eight of these strains the concentrations of BA were found to be instrumentally non-detectable (data not shown). The percentage of positive strains on the total number of strains was in agreement with results reported by other authors [15].

Selected strains with non-active carboxylase and high acidogenic power were tested for their fermentative strength, in meat model, evaluating the decrease of pH and the sensorial characteristic

after 72 hours of fermentation under vacuum at 15°C. Out of 8 tested strains, *L. plantarum* VLT73 was found to be the most suitable. It did not influence the organoleptic profile of the meat and confirmed a decrease of pH to 5.40 after 24 hours of incubation. The next step of this experimentation involved the use of this strain as starter culture in order to produce different batches of Felino style salami.

5.4.2 Microbial count.

At time zero meat mixtures without inoculum (batches G, H, I) contained low level of LAB (less than 10 CFU/g) while in the inoculated batches LAB reached more than 10^7 of CFU/g, confirming the effectiveness of the added starter (Table 2).

Table 2: pH and microbial counts (log CFU/g) during ripening. Means ± standard deviation. (nd=not detected)

Batch	Starter cultures[a]	Preservative[b]	Ripening (d)	pH	LAB	Gram+coag-cocci	*Enterococci*	*Entero-bacteriaceae*
A	L + K	$NaNO_3$	0	5.56 ± 0.06	7.07 ± 0.11	4.61 ± 0.13	<1.00	2.23 ± 0.18
			3	5.40 ± 0.06	8.57 ± 0.22	4.42 ± 0.09	5.01 ± 0.13	2.01 ± 0.22
			10	5.22 ± 0.07	8.55 ± 0.22	5.83 ± 0.38	4.52± 0.23	2.48 ± 0.15
			24	5.37 ± 0.18	8.42 ± 0.09	3.05 ± 0.08	4.44± 0.45	1.71 ± 0.12
			45	5.77 ± 0.22	8.53 ± 0.12	4.70 ± 0.14	3.52± 0.55	0.99 ± 0.23
			60	6.06 ± 0.07	7.76 ± 0.10	6.10 ± 0.12	3.66± 0.10	<1,00
B	L + K	$NaNO_2$	0	5.51 ± 0.01	7.17 ± 0.09	5.06 ± 0.10	<1.00	2.17 ± 0.05
			3	5.38 ± 0.03	8.38 ± 0.09	4.01 ± 0.13	<1.00	<1.00
			10	5.22 ± 0.02	8.37 ± 0.08	5.75 ± 0.13	<1.00	<1.00
			24	5.32 ± 0.19	8.44 ± 0.16	3.58 ± 0.25	2.45± 0.16	<1.00
			45	5.65 ± 0.20	8.35 ± 0.19	5.46 ± 0.14	2.31± 0.13	<1.00
			60	5.87 ± 0.08	8.36 ± 0.19	5.27 ± 0.16	2.32 ± 0.17	<1.00
C	L + K	None	0	5.53 ± 0.08	7.20 ± 0.11	4.76 ± 0.23	<1.00	1.98 ± 0.11
			3	5.43 ± 0.04	8.48 ± 0.05	4.08 ± 0.19	5.07 ± 0.22	2.47 ± 0.23
			10	5.21 ± 0.03	8.43 ± 0.10	5.07 ± 0.15	4.08 ± 0.05	<1.00
			24	5.33 ± 0.18	8.51 ± 0.08	3.48 ± 0.16	4.02 ± 0.09	<1.00
			45	5.43 ± 0.18	8.45 ± 0.06	4.54 ± 0.23	4.00 ± 0.18	<1.00
			60	5.92 ± 0.07	8.20 ± 0.20	5.03 ± 0.13	4.66 ± 0.12	<1.00
D	L	$NaNO_3$	0	5.63 ± 0.01	7.16 ± 0.05	3.01 ± 0.22	<1.00	2.37 ± 0.18
			3	5.42 ± 0.09	8.33 ± 0.04	3.68 ± 0.10	4.80 ± 0.15	2.30 ± 0.23
			10	5.32 ± 0.24	8.47 ± 0.05	5.71 ± 0.44	4.80 ± 0.18	2.30 ± 0.30
			24	5.43 ± 0.19	8.48 ± 0.05	3.52 ± 0.39	3.79 ± 0.32	1.49 ± 0.12
			45	6.00 ± 0.18	8.77 ± 0.13	5.52 ± 0.26	3.10 ± 0.12	0.98 ± 0.08
			60	5.98 ± 0.05	8.45 ± 0.31	5.52 ± 0.51	3.75 ± 0.23	<1.00
E	L	$NaNO_2$	0	5.58 ± 0.06	7.12 ± 0.11	3.19 ± 0.18	<1.00	2.45 ± 0.12
			3	5.56 ± 0.06	7.66 ± 0.11	3.72 ± 0.25	<1.00	1.98 ± 0.08
			10	5.32 ± 0.11	9.09 ± 0.42	5.95 ± 0.05	4.39 ± 0.15	1.98 ± 0.09
			24	5.42 ± 0.09	8.04 ± 0.08	3.63 ± 0.21	2.76 ± 0.07	1.46 ± 0.10
			45	5.78 ± 0.17	8.43 ± 0.22	5.38 ± 0.14	2.69 ± 0.27	<1.00
			60	5.63 ± 0.06	7.93 ± 0.22	5.58 ± 0.13	3.50 ± 0.13	<1.00
F	L	None	0	5.49 ± 0.06	7.08 ± 0.03	3.23 ± 0.12	<1.00	2.23 ± 0.27
			3	5.45 ± 0.06	7.85 ± 0.05	4.36 ± 0.18	4.50 ± 0.43	1.95 ± 0.10
			10	5.22 ± 0.05	8.37 ± 0.25	6.03 ± 0.60	4.97 ± 0.12	2.00 ± 0.23
			24	5.24 ± 0.06	8.45 ± 0.22	3.44 ± 0.52	3.73 ± 0.11	0.98 ± 0.09
			45	5.53 ± 0.14	8.88 ± 0.19	5.30 ± 0.18	4.91 ± 0.19	<1.00
			60	5.70 ± 0.07	8.36 ± 0.28	4.76 ± 0.15	4.72 ± 0.17	<1.00
G	N	$NaNO_3$	0	5.61 ± 0.04	n.d.	3.13 ± 0.29	<1.00	2.37 ±
			3	5.63 ± 0.04	7.86 ± 0.05	3.96 ± 0.17	5.46 ± 0.02	2.87 ±
			10	5.29 ± 0.05	8.51 ± 0.40	5.43 ± 0.22	5.71 ± 0.11	<1.00
			24	5.52 ± 0.22	8.52 ± 0.40	4.01 ± 0.19	5.58 ± 0.47	1.23 ±
			45	5.86 ± 0.16	8.36 ± 0.26	5.39 ± 0.37	5.09 ± 0.38	1.99 ±
			60	5.90 ± 0.07	7.58 ± 0.22	5.74 ± 0.29	5.08 ± 0.30	<1.00
H	N	$NaNO_2$	0	5.62 ± 0.06	n.d.	3.21 ± 0.40	<1.00	2.47 ± 0.34
			3	5.62 ± 0.09	7.13 ± 0.09	3.39 ± 0.14	<1.00	2.68 ± 0.45
			10	5.39 ± 0.08	8.12 ± 0.12	3.65 ± 0.20	4.01 ± 0.15	1.01± 0.10
			24	5.38 ± 0.10	7.80 ± 0.16	2.98 ± 0.13	3.60 ± 0.15	1.00± 0.03
			45	5.77 ± 0.13	7.93 ± 0.09	4.77 ± 0.12	3.25 ± 0.12	<1.00
			60	6.03 ± 0.06	6.65 ± 0.36	5.11 ± 0.30	3.49 ± 0.17	<1.00
I	N	None	0	5.60 ± 0.03	n.d.	3.23 ± 0.38	<1.00	2.18 ± 0.01
			3	5.63 ± 0.05	7.64 ± 0.18	3.04 ± 0.25	5.41 ± 0.13	2.60 ± 0.12
			10	5.38 ± 0.06	7.79 ± 0.31	3.17 ± 0.26	5.74 ± 0.22	4.45 ± 0.45
			24	5.55 ± 0.21	7.85 ± 0.17	3.41 ± 0.26	5.37 ± 0.32	<1.00
			45	5.74 ± 0.16	8.02 ± 0.07	5.65 ± 0.19	5.06 ± 0.37	2.58 ± 0.23
			60	6.34 ± 0.05	7.50 ± 0.41	5.45 ± 0.75	5.12 ± 0.40	<1.00

[a] Starter cultures: L = *Lactobacillus plantarum* VLT 73; K = *Kocuria varians* MIAL; N= none

[b] Preservative: $NaNO_3$= Sodium nitrate 250 mg/kg; $NaNO_2$ = Sodium nitrie 150 mg/kg

In these cases, fermentation was quick and effective, bringing the pH to levels lower than 5.5 after 3

days. In batches G, H and I, (no inoculation), LAB were also present after 3 days with values higher than 10^7 CFU/g, but the acidification was slower and less effective. These results confirmed again that short acidification times are mainly due to the use of starter cultures and therefore it is a necessary step to produce safer higher quality products.. Batches in which nitrates and nitrites were added did not show particular differences in microbial count, proving that their use does not affect the development of LAB [9, 20].

Gram positive - coagulase negative cocci, counted on MSA, showed a similar behaviour to that of lactic acid bacteria. In non-inoculated batches (G,-I) or in batches inoculated with only *L. plantarum* 32 (D-F), around 10^3 log CFU/g were present, while counts in batches inoculated with *K. varians* MIAL12 (A-C) were 1.5 logarithmic cycles higher at least. This difference was particularly appreciated in the first 3 weeks of ripening; while in the subsequent steps of ripening counts decreased, reaching the Microbial count.same level for all the batches. This result is in agreement with Bover-Cid et al. [21].

Enterococci, evaluated in Slanetz and Bartley Agar, were particularly influenced by the presence of nitrites and nitrates. At time zero, these microorganisms were present with amount lower than 10 CFU/g; after only three days they reached values higher than 10^5 CFU/g in batches without the addition of nitrites, maintaining this level in the other steps of the ripening. The strong inoculation of lactobacilli allowed a partial control of the proliferation of enterococci; however their level reached values comparable with non inoculated batches. In batches B, E, and H (with addition of nitrites), the level of enterococci increased only after the 10^{th} day of ripening, reaching a stable level of about 10^3 CFU/g.

Enterobacteriaceae and enterococci had a similar behaviour: in batches B and E, (addition of nitrites and inoculation of lactic acid bacteria) their level decreased to 100 CFU/g after 3 days. This level was reached in the other batches only in subsequent steps of the ripening. Other authors [9, 21] have also suggested that nitrites and lactic acid bacteria started cultures can be used to control *Enterobacteriaceae* since these microorganisms are the main factors in the production and

accumulation of biogenic amines in salami.

5.4.3 Formation of biogenic amines

Spermine (37.3 ± 3.45 mg/kg) and spermidine (12.5 ± 2.23 mg/kg) were the only BA detectable in the meat mixture at time zero; the other BA were present in trace. Table 3 details the concentration of biogenic amines at the end of the ripening (60 days).

Table 3 – concentration of biogenic amine (mg/kg) at the end of ripening process (60 days).

		Preservative		
Amine	Starter culture	$NaNO_2$ (150 mg/kg)	$NaNO_3$ (250 mg/kg)	None
Putrescine	*Lactobacillus* + *Kocuria*	$11.1^{a1} \pm 2.0$	$193.8^{3} \pm 7.2$	$116.4^{a2} \pm 11.1$
	Lactobacillus	$177.8^{b} \pm 3.8$	220.4 ± 39.4	$222.5^{c} \pm 8.1$
	none	$216.5^{b} \pm 22.3$	183.7 ± 14.2	$159.4^{b} \pm 1.9$
Cadaverine	*Lactobacillus* + *Kocuria*	$44.0^{b1} \pm 4.4$	$67.7^{ab2} \pm 1.4$	$59.5^{b12} \pm 6.8$
	Lactobacillus	$10.5^{a1} \pm 1.0$	$55.9^{a3} \pm 0.9$	$42.3^{a2} \pm 0.8$
	none	$16.9^{a1} \pm 1.0$	$80.5^{b2} \pm 5.9$	$80.2^{c2} \pm 0.9$
Tryptamine	*Lactobacillus* + *Kocuria*	$2.8^{a1} \pm 0.6$	$14.3^{a2} \pm 0.3$	$17.6^{a2} \pm 2.4$
	Lactobacillus	$7.1^{b1} \pm 0.4$	$26.6^{b2} \pm 0.0$	$28.0^{b2} \pm 1.1$
	none	$12.4^{c1} \pm 0.5$	$32.8^{c2} \pm 0.7$	$34.2^{b2} \pm 2.2$
Spermidine	*Lactobacillus* + *Kocuria*	$2.2^{a1} \pm 0.5$	$26.7^{a2} \pm 4.8$	$18.5^{a2} \pm 3.7$
	Lactobacillus	$22.1^{b1} \pm 2.0$	$34.1^{a2} \pm 2.2$	$30.5^{a2} \pm 1.2$
	none	$48.6^{c1} \pm 4.7$	$83.2^{b2} \pm 1.3$	$98.6^{b3} \pm 4.1$
Spermine	*Lactobacillus* + *Kocuria*	41.2 ± 7.4	59.6 ± 11.3	49.8 ± 0.6
	Lactobacillus	51.6 ± 2.5	57.6 ± 0.1	52.8 ± 6.1
	none	50.1 ± 0.0	36.6 ± 6.8	38.2 ± 2.7
Histamine	*Lactobacillus* + *Kocuria*	54.6 ± 2.1	$60.0^{ab} \pm 5.0$	64.0 ± 3.7
	Lactobacillus	54.6 ± 7.1	$94.4^{b} \pm 10.5$	60.3 ± 12.4
	none	46.0 ± 0.5	$37.4^{a} \pm 5.3$	61.1 ± 26.9
Tyramine	*Lactobacillus* + *Kocuria*	$19.0^{a1} \pm 4.6$	$108.0^{2} \pm 6.0$	$43.8^{1} \pm 23.2$
	Lactobacillus	$95.0^{c2} \pm 1.9$	$86.6^{12} \pm 3.6$	$79.5^{1} \pm 1.5$
	none	$60.9^{b} \pm 7.9$	85.9 ± 17.1	43.4 ± 5.3
Total Amine	*Lactobacillus* + *Kocuria*	$174.7^{a1} \pm 12.5$	$530.0^{a2} \pm 35.9$	$369.8^{a3} \pm 2.1$
	Lactobacillus	$418.7^{b1} \pm 11.2$	$568.1^{a2} \pm 50.4$	$515.9^{b12} \pm 12.0$
	none	$451.4^{b} \pm 25.5$	$540.2^{a} \pm 51.2$	$515.1^{b} \pm 13.6$

Means ± standard deviations. For each amine, any means followed by different superscript show statistically differences ($p < 0.05$) according to the *post hoc* comparisons (Tukey's HSD) of the ANOVA. Letters compare among the different starter cultures, numbers among preservatives.

The most prevalent amines were putrescine and tyramine, particularly in batches without addition of starter culture and preservatives. This is in agreement with previous works [7, 22] in particular for traditional Spanish salami [12, 23, 24].

As evidenced by the results, the use of nitrite preservatives can control the formation of biogenic amines; in particular, the amounts of putrescine, cadaverine, tryptamine and tyramine showed statistically significant differences ($p<0.05$) if compared with control samples and salami with nitrate preservatives.

Use of nitrites enabled the control of the production of putrescine in batch B which also involved the use of a starter culture composed of 2 strains; however they were less effective when the *Kocuria* strain was not inoculated ($p<0.05$). The production of putrescine, which was the most prevalent BA in all the samples, was influenced by the presence of nitrites and by the inoculation of the *Kocuria* strain. Previously Gardini et al. [10], evidenced similar behaviour, demonstrating the ability of a *Staphylococcus xylosus* strain to maintain the concentration of putrescine at level 30 times lower than the control sample. Data related to putrescine are confirmed by the decreased bacterial charge of *Enterobacteriaceae* [25] and faecal enterococci [7] in batches that showed the lower content in putrescine.

Moreover, the use of nitrites allowed for tryptamine to be limited to levels lower than 10 mg/kg for the batches with inoculation; in control non-inoculated sample this amine was found in amount of 12.42 mg/kg [26]. Differences in tryptamine concentration was statistically significant between batches produced with different starters for the inoculation, and also if salami were produced with or without nitrates.

Data related to cadaverine showed that the production of this biogenic amine is reduced by the presence of nitrites, but in this case, inoculation with *Kocuria* was not able to decrease its production. Moreover, salami with *Kocuria* showed concentrations of cadaverine that were significantly higher than those inoculated only with the *Lactobacillus* strain. The counts of enterococci, which were not influenced by the presence of *Kocuria* as starter culture, could explain

these results.

The concentration of spermidine was also influenced by the use of nitrites, in the same way as the other amines. Concentrations of this amine in salami with the combination of the two microorganisms in the starter culture were always significantly smaller when compared with non-inoculated control samples. The use of preservatives and the different combination of the starter culture did not affect the amounts of spermine which maintained its level in a range between 36 an 59 mg/kg. Other works [23] have indicated that spermine and spermidine are already present in meat during mixing. These results demonstrate that is not possible to control the production of these two biogenic amines with technological coadiuvant as preservatives or starter cultures [13, 27].

Histamine, found in all the samples, did not show significant differences between the batches with nitrites and without preservatives. However batches with nitrates confirmed the results observed by other authors [10, 28], the amount of histamine decreased in batches with starter culture. These authors suggested the use of *Staphylococcus xylosus* to control the production of biogenic amines. Moreover, the strict relation between the accumulation of histamine during ripening (data not shown) and the charge of faecal enterococci must be underlined, as reported by other authors [29].

Accumulation of tyramine is linked with the proliferation of lactic acid bacteria, while the presence of sugars added to meat allows control of their production [29]. The concentration of this amine, however, is in agreement with previous results obtained by these authors in salami produced with added sugars. The use of nitrites allowed the control of amines to levels lower than 200 mg/kg in the batches with the use of *Lactobacillus* and *Kocuria* as starter culture.

Table 4 reports Pearson's correlation coefficient between the counts of *Enterobacteriaceae* and enterococci and BA amount.

Table 4 Correlation coefficients between biogenic amines and microbial counts

	Enterobacteriaceae	Enterococci
Putrescine	-0.275**	0.376**
Cadaverine	-0.099	0.341**
Tryptamine	-0.085	0.408**
Spermidine	-0.188**	0.247**
Spermine	0.185**	0.071
Histamine	-0.349**	0.016
Tyramine	-0.213*	0.161
Total amine	-0.225*	0.322**

** $p < 0.01$
* $p < 0.05$

It can be seen that a strongly significant correlation ($p<0.01$) exists between the count of *Enterobacteriaceae* and the amounts of putrescine, spermine, spermidine and histamine. A correlation with lower but consistent significance ($p<0.05$) was observed between the counts of *Enterobacteriaceae* and tyramine. The total amount of amines showed a less significant relation with the counts of *Enterobacteriaceae*, while it resulted more significantly influenced by the counts of enterococci. At the same time a significant relation between these counts and the concentration of putrescine, cadaverine, tryptamine and spermidine was found.

5.4.4 Sensorial Characteristics

A trained panel carried out sensorial analysis on all the batches previously produced, at the end of ripening. The analysis of batches produced with the same starter (Figure 1) showed that the use of nitrites improved the structural characteristics as hardness, elasticity and masticability. The influence of the starter culture for these salami is limited; in fact, the sensorial profiles of thesis B, E and H were more or less the same.

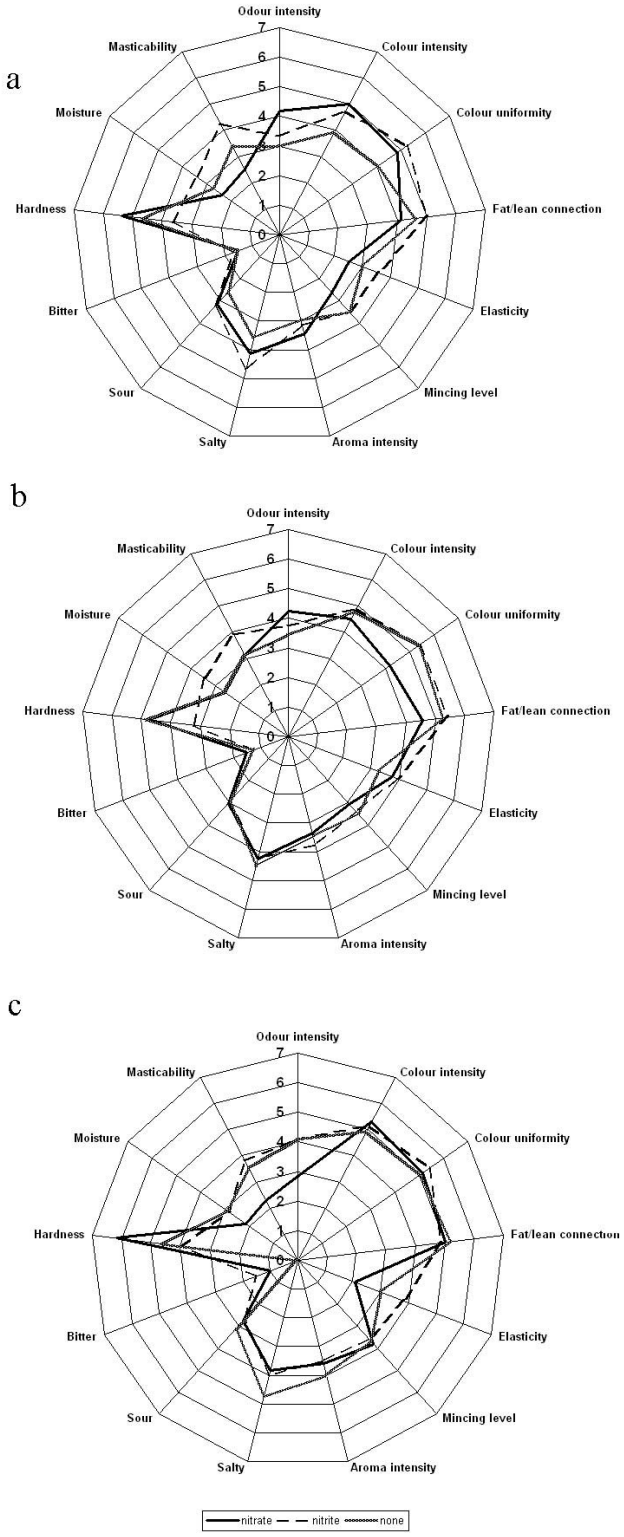

Fig.1 Sensory profile of batches obtained. a) batches with addition of *Lactobacillus plantarum* VLT73 and *Kocuria varians* MIAL 12; b) batches with *Lactobacillus plantarum* VLT73; c) batches without addition of starter cultures.

The starter cultures used in this work notably influenced the sensorial characteristics of salami with added nitrates (A, D and G) or salami without preservatives (C, F and I). The role of *Kocuria varians* was appreciable particularly in term of colour when compared with salami produced using

only *L. plantarum* or without starter. *L. plantarum* evidenced its own characteristics only in batches with added nitrates. Use either cultures gave rise to the perception of a slight bitter taste in comparison to those produced without starter culture.

The results obtained clearly show that the use of nitrates and, especially nitrites, when combined with the use of selected starter culture (as lactic acid bacteria from *Lactobacillus* genus and Gram positive - coagulase negative cocci *Kocuria* genus) generate safer salami products with an improved sensorial profile. The disciplinary of production of PDO salami should take into account this information with strong qualitative and hygienic-sanitary concern.

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6 RETENTION EFFECTS OF OXIDIZED POLYPHENOLS DURING ANALYTICAL EXTRACTION OF PHENOLIC COMPOUNDS OF VIRGIN OLIVE OIL

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6.1 Abstract

The hydrophilic extract of virgin olive oil contains several phenolic compounds such as simple phenols, lignans and secoiridoids that have been widely studied in recent years. Interest in the hydrophilic extract has also been extended to the fraction of oxidized phenols that form during storage as a consequence of oxidative stress. The present investigation compares the two most commonly used extraction methods, namely liquid-liquid extraction and solid-phase extraction, on fresh virgin olive oil and that kept at different temperatures in the presence of oxygen to promote the formation of oxidative products. The selective retention of these natural and oxidized phenolic compounds in relation to the extraction method was assessed. Quantification of eight identified phenolic molecules and 11 unknown peaks was performed by HPLC-DAD/MSD.

Keywords: Virgin olive oil, Oxidized phenolic compounds, Extraction, HPLC.

Non-standard Abbreviations: 3,4-dihydroxyphenyl-ethanol (3,4-DHPEA); 3,4-dihydroxyphenyl-ethanol linked to elenolic acid (3,4-DHPEA-EA); 3,4-dihydroxyphenyl-ethanol linked to dialdehydic form of elenolic acid (3,4-DHPEA-EDA); 1-acetoxypinoresinol (Ac Pin); Liquid-Liquid- extraction (LLE); *p*-hydroxyphenyl-ethanol (*p*-HPEA); *p*-hydroxyphenyl-ethanol linked to elenolic acid (*p*-HPEA-EA); *p*-hydroxyphenyl-ethanol linked to dialdehydic form of elenolic acid (*p*-HPEA-EDA); pinoresinol (Pin); Solid-Phase Extraction (SPE); Total Peak Area Ratio (TPAR).

6.2 Introduction

Investigations regarding natural antioxidants from vegetable matrices have shown that olives and olive-derivatives are an important part of the Mediterranean diet and, along these lines, are now as a source of natural phenolic antioxidants [1,2]. These compounds have documented chemoprotective properties in human beings, especially in the prevention of cardiovascular diseases [3-5], and also to contribute to the sensorial properties of virgin olive oils by conferring bitterness, pungency and astringency [6-8]. Moreover, the high oxidative stability of virgin olive oil is related not only to the high monounsatured/polyunsatured ratio, but also to the presence of phenolic compounds with antioxidant action.

The hydrophilic extract of virgin olive oil contains several phenolic compounds including simple phenols, lignans and secoiridoids [9-12]. However in order to determine the actual amount of phenols in olive oil, it is important utilise an analytical method that provides adequate recoveries in the extraction phase. Two basic techniques have been generally employed for the extraction of phenolic compounds from virgin olive oil, namely solid phase extraction (SPE) and liquid-liquid extraction (LLE). Previous investigations have determined the recoveries of LLE and SPE by assaying for the different stationary phases (C_8, C_{18}, Diol) and several elution mixtures [11, 13, 14].

During the last five years, the interest in oxidized phenolic compounds has increased significantly, especially in relation to determining the freshness/ageing status of virgin olive oil [15, 16] or thermal treatments [17]. In particular, the most recent reports have focused on the oxidized phenolic compounds produced by thermic and forced/spontaneous oxidative stress, as in the case of extended conservation. However the extraction method has not been evaluated and the same methods usually employed for the analysis of phenol in fresh oil have been systematically used. Accurate characterization of these oxidative compounds could represent an analytical instrument to investigate the thermal processes that oils undergo during refinement, and could also provide a mean to verify moderately refining practices when used with “fraudulent” purposes that polish up

non-irreprehensible oils (generally defined gentle deodorization) or blend virgin olive oil with other oils. The aim of this work was to evaluate the influence of the two most widely used extraction methods for separation of natural and oxidized phenolic compounds on oil samples subjected to accelerated oxidation by either increased temperature or natural oxidation under different storage conditions.

6.3 Experimental

6.3.1 Reagents

Methanol, *n*-hexane, ethyl acetate, HPLC-grade water and HPLC-grade methanol were purchased from Merck, syringic acid was acquired from Fluka (Buchs, Swiss), Diol-SPE columns 500 mg/3 ml were from Isolute, International Sorbent Technologies Ltd. (Hengoed, UK), and 0.2 μm nylon filters were purchased from Whatman Inc. (Florham Park, NJ, USA).

6.3.2 Samples

Thermal oxidation

A virgin olive oil produced from olives of Cornicabra cultivar in Spain during crop season 2005 with a 2-phase industrial olive mill was the non-oxidised reference sample. Approximately 1000 mL of sample was distributed into three 500 mL bottles without a stopper. One three-bottle batch was stored in an oven at 50 ºC and a second batch at 80 ºC for 3 months. Phenolic compounds were extracted by both SPE and LLE (see below). Extracts 0LLE and 0SPE were obtained from the fresh sample analyzed immediately, 350LLE and 350SPE from the same sample maintained 3 months at 50° C, and 380LLE and 380SPE from that maintained 3 months at 80° C.

Natural oxidation

This experimental phase involved 3 different virgin olive oils exposed natural oxidation:

Sample A: supermarket sample produced in 2003, stored 31 months closed at room temperature, in the dark with high headspace.

Sample B: D.O.P. Chianti Classico produced in 2004, stored 19 months at room temperature under

natural light and without a stopper to permit photo-oxidation and continuous contact with oxygen.

Sample C: Produced by Nostrana di Brisighella cv. in 2005 and stored 7 months with a stopper in the dark at 9°C.

The LLE and SPE methods were compared for all three samples.

6.3.3 Solid-Phase extraction

As described by Gómez-Alonso et al. [18], 250 µL volume of the internal standard (15 mg/L syringic acid in methanol) was added to a sample of virgin olive oil (2.5 g) and vortexed for one minute. The solvent was evaporated with a rotary evaporator at 35 °C under vacuum. The oil was then dissolved in 6 ml of *n*-hexane and a diol-bonded phase cartridge was used to extract the phenolic fraction. The cartridge was conditioned with methanol (6 mL) and *n*-hexane (6 mL), the oil solution was then applied, and the SPE column was washed with *n*-hexane (3 mL twice) and then with *n*-hexane/ethyl acetate (85:15, v/v; 4 mL). Finally, the phenols were eluted with methanol (15 mL) and the solvent was removed with a rotary evaporator at 35 °C under vacuum to dryness. The phenolic residue was dissolved in methanol/water (1:1 v/v; 250 µL) and filtered through a 0.2 µm nylon filter before analysis by HPLC.

6.3.4 Liquid-Liquid extraction

The phenolic fraction was extracted from the oil with a liquid/liquid extraction method described by Pirisi *et al.* (2000) [19]. Two grams of oil was weighed in a centrifuge tube and the internal standard (200 µl of a 15 mg/L solution of syringic acid in methanol) along with 1.0 mL of *n*-hexane and 2.0 mL of methanol/water (v/v, 60/40) were added. The mixture was stirred for 2 min in a vortex apparatus, and the tube was centrifuged at 1490g. The methanol layer was separated and the extraction repeated twice. The extracts were combined and washed twice with 2 mL of *n*-hexane. The *n*-hexane was discarded, and the methanolic solutions were vacuum dried at low temperature (<35 °C). The dry extracts were redissolved in 200 µL of methanol/water (1:1, v/v) and filtered through a 0.2 µm nylon filter before HPLC. Moreover, a LLE on sample B was also carried out; the

extract was loaded in the diolic SPE column, and eluted with methanol (without washing steps) to evaluate the effect of retention without matrix interaction. This extract was indicated as extract D.

6.3.5 Primary and secondary auto-oxidation products

Evaluation of primary auto-oxidation products was carried out by determination of the peroxide value (PV) according to the official methods described in European Regulation EEC 2568/91 and the following amendments [20]. PV was expressed as meq O_2/kg of oil.

Determination of oxidized fatty acids (OFA) was carried out on samples naturally oxidized according to Rovellini and Cortesi [21, 22] by HPLC-DAD analyses after transesterification with 1.0 M sodium benzyloxyde in benzyl alcohol; the chromatograms were recorded at 255 nm. The results were expressed as percentage considering benzyl heptadecanoate as an internal standard. These compounds were tentatively identified basing on their UV-VIS and mass spectra obtained by HPLC-DAD/ESI-MSD and comparison with literature data.

6.3.6 HPLC determination of phenolic compounds

HPLC analysis was performed using an HP 1100 Series instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump delivery system, degasser, autosampler, diode array UV-Vis detector (DAD) and mass spectrometer detector (MSD). The instrument was equipped with a reverse phase C_{18} Luna™ column (5 µ m, 25 cm x 4.60 mm ID; Phenomenex, Torrence, CA, USA); an injection volume of 10 µL and a flow rate of 1.0 mL/min were utilised. The mobile phase was a mixture of water/acetic acid (95:5 v/v) (solvent A), and methanol/acetonitrile (50:50 v/v) (solvent B). The gradient, in % solvent B, changed from 5% to 66% in 50 min, from 66% to 100% in 2 min, remained at 100% for 13 min, decreased to 5% in 3 min and then remained at 5% for 4 min. The total run time was 72 min. Phenolic compounds were tentatively identified based on their UV-Vis and mass spectra obtained by HPLC-DAD/ESI-MSD and comparison with literature data [23].

6.4 Results

As seen in Figure 1 and Table 1, there were no striking differences between the extracts of fresh samples 0LLE and 0SPE in agreement with previous reports [14, 24].

Figure 1

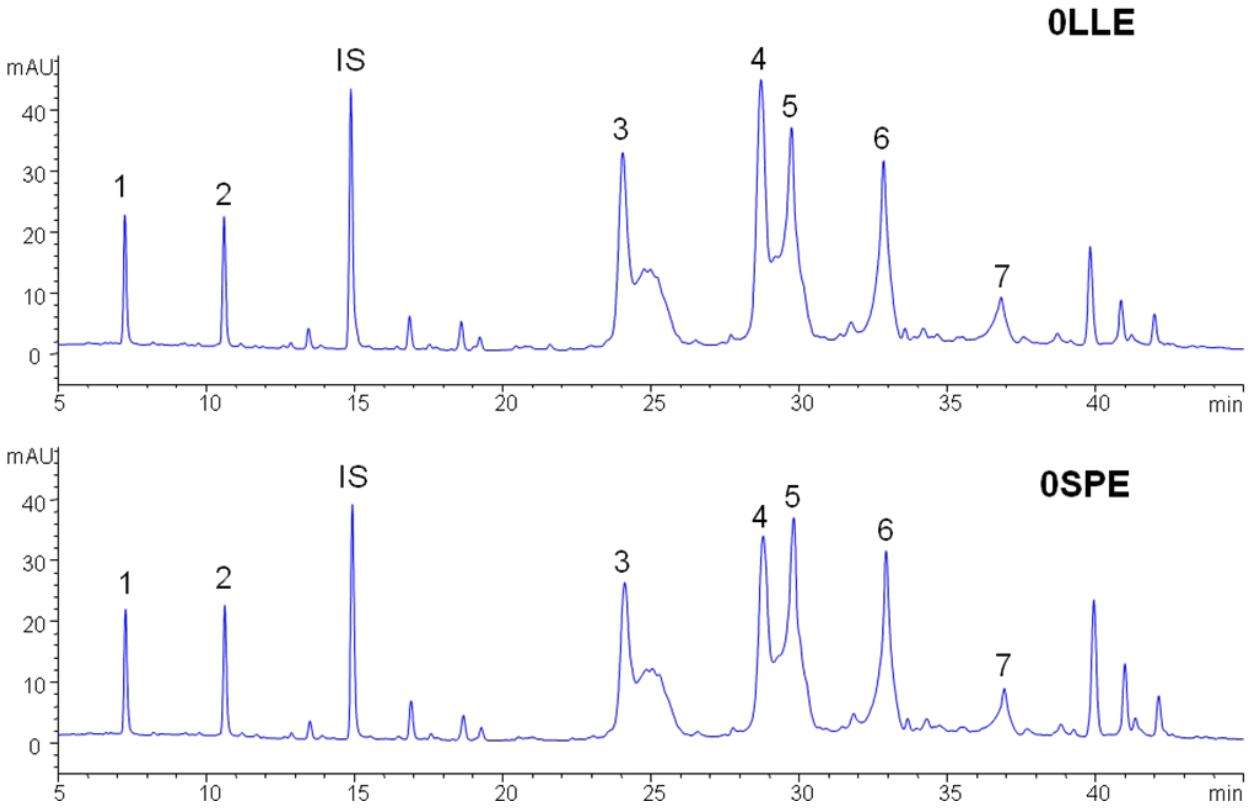

Figure 1: Chromatograms of fresh samples 0LLE and 0SPE. 1: 3,4-DHPEA; 2: *p*-HPEA; 3: 3,4-DHPEA-EDA; 4: *p*-HPEA-EDA; 5: Pin + Ac Pin; 6: 3,4 - DHPEA-EA; 7: *p*-HPEA-EA; IS: internal standard.

Table 1

Table 1

	UV spectra maxima (nm)	0LL X ± SD	0SPE X ± SD	350LL X ± SD	350SPE X ± SD	380LL X ± SD	380SPE X ± SD
3,4-DHPEA	236/280	41.7 ± 0.3 (a)	46.8 ± 1.0 (a)	67.1 ± 3.6 (a)	71.8 ± 0.6 (a)	nd	nd
p-HPEA	234/276	42.4 ± 2.6 (a)	52.4 ± 1.0 (a)	118 ± 7 (a)	125 ± 2 (a)	47.8 ± 1.8 (b)	65.6 ± 2.9 (a)
3,4-DHPEA-EDA	236/282	432 ± 31 (a)	483 ± 43 (a)	73.9 ± 6.1 (a)	70.6 ± 2.1 (a)	nd	nd
p-HPEA-EDA	236/276	248 ± 32 (a)	277 ± 42 (a)	197 ± 18 (a)	155 ± 3 (b)	12.6 ± 1.6	nd
Pin + Ac Pin	236/278	228 ± 42 (b)	323 ± 1 (a)	142 ± 21 (a)	144 ± 5 (a)	36.6 ± 3.9	nd
3,4 - DHPEA-EA	238/280	189 ± 32 (a)	229 ± 1 (a)	45.9 ± 5.1 (a)	43.4 ± 1.1 (a)	nd	nd
p-HPEA-EA	236/276	44.4 ± 7.8 (a)	48.5 ± 2.5 (a)	33.9 ± 14.8 (a)	32.6 ± 0.9 (a)	26.8 ± 0.9	nd
Unknown 1 Rt=12,91	268	nd	nd	75.3 ± 4.1 (a)	83.3 ± 0.6 (a)	nd	nd
Unknown 2 Rt=15,01	236/302	nd	nd	40.8 ± 0.3 (a)	49.4 ± 8.4 (a)	nd	nd
Unknown 3 Rt=17,83	274	nd	nd	68.6 ± 7.7 (a)	73.7 ± 1.7 (a)	nd	nd
Unknown 4 Rt=29,29	236/276	nd	nd	65.6 ± 5.0 (a)	60.8 ± 0.6 (a)	nd	nd
Unknown 5 Rt=13,90	262/290	nd	nd	nd	nd	17.1 ± 0.4	nd
Unknown 6 Rt=14,31	284	nd	nd	nd	nd	26.7 ± 0.7	nd
Unknown 7 Rt=21,83	236/280	nd	nd	nd	nd	128 ± 4 (a)	47.5 ± 4.8 (b)
Unknown 8 Rt=32,32	236/274	nd	nd	nd	nd	79.0 ± 10.8	nd
Unknown 9 Rt=34,29	232/276	nd	nd	nd	nd	57.8 ± 3.4	nd
Unknown 10 Rt=35,02	236/280	nd	nd	nd	nd	49.8 ± 0.7	nd
Unknown 11 Rt=35,90	236/280	nd	nd	nd	nd	88.6 ± 4.9	nd

Quantification of peaks respect to the internal standard

$X = A_x / A_{IS} \cdot 100$, where A_x is the area of single peaks and A_{IS} is the area of the internal standard, and X is the mean value of $n = 2$ repetitions; nd: not detected.

a-b Same letters within each row at same condition (0, 350 or 380) do not significantly differ ($p \leq 0.05$).

Moreover, samples 350LLE and 350SPE, kept for 3 months at 50° C, did not show significant quantitative differences with respect to the internal standard (Table 1). However for these samples as is clearly evident in the tracings in Figure 2, the areas of the single peaks are reduced in samples subjected to SPE. This is even more evident in the expression of total peak area ratio (TPAR) between SPE and LLE extracts (Table 2).

Figure 2

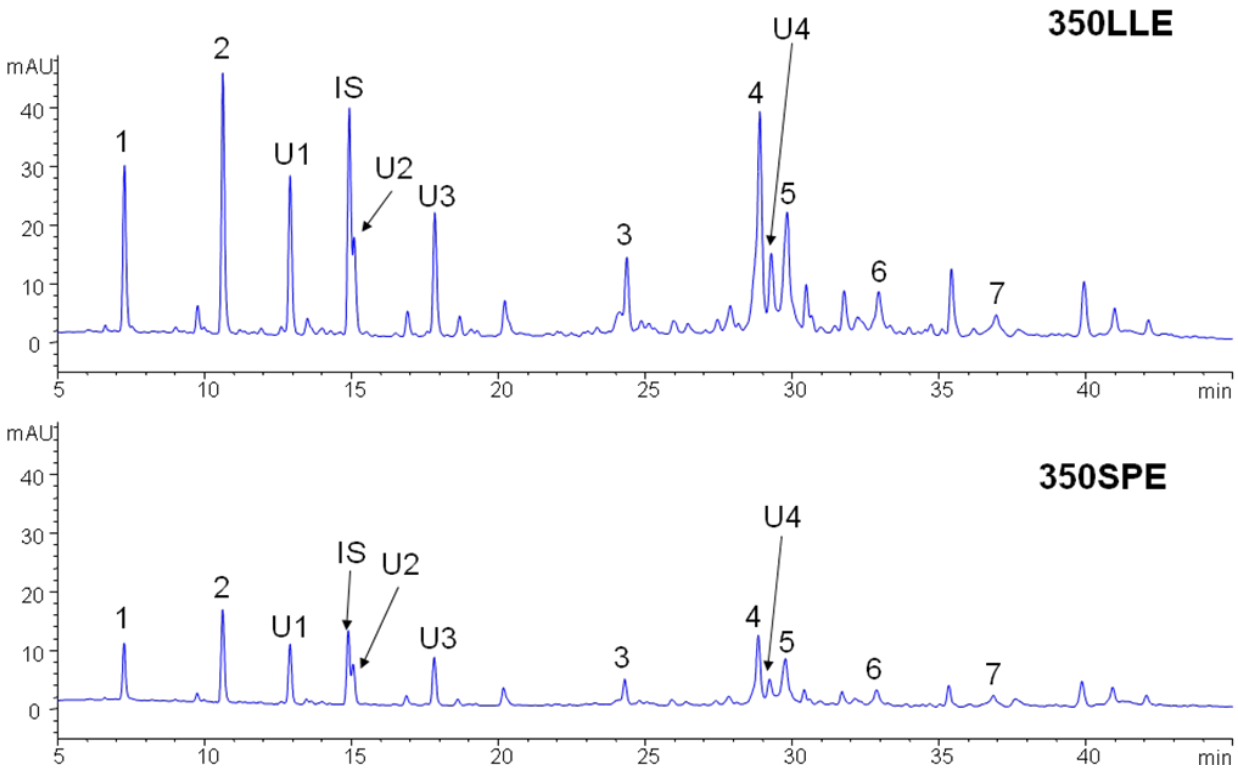

Figure 2: Chromatograms of samples 350LLE and 350SPE maintained at 50° C for 3 months. 1: 3,4-DHPEA; 2: *p*-HPEA; 3: 3,4-DHPEA-EDA; 4: *p*-HPEA-EDA; 5: Pin + Ac Pin; 6: 3,4 - DHPEA-EA; 7: *p*-HPEA-EA; IS: internal standard. U1-U4: unknown peaks.

Table 2

Table 2

	Total Peak Area	TPAR
00LL	4523.52	1.24
0SPE	5623.29	
350350LL	4237.86	0.41
350SPE	1728.33	
380380LL	3980.07	0.08
380SPE	329.26	

Artificially oxidized samples. 0: fresh sample; 350: sample kept at 50° C for 3 months; 380: sample kept at 80° C for 3 months. TPAR is the Total Peak Area Ratio obtained from SPE/LLE values of the Total Peak Area of samples subjected to the same storage conditions.

In fresh samples this ratio was 1.24, while in the samples kept for 3 months it was decreased to 0.41. The TPAR was further reduced to a value of 0.08 in sample 380, which presumably caused greater oxidation of phenolic compounds after conservation for 3 months at 80° C. The results of the two extraction methods on fresh samples and on samples maintained at 50° C were comparable

in terms of the quantification of single peaks with respect to the internal standard; however this was not seen in the samples kept at 80° for 3 months (Table 1). In fact, extract 380SPE underwent a drastic reduction of the total peak area and a strong decrease of the quantifiable peaks (Fig. 3, Table 2).

Figure 3

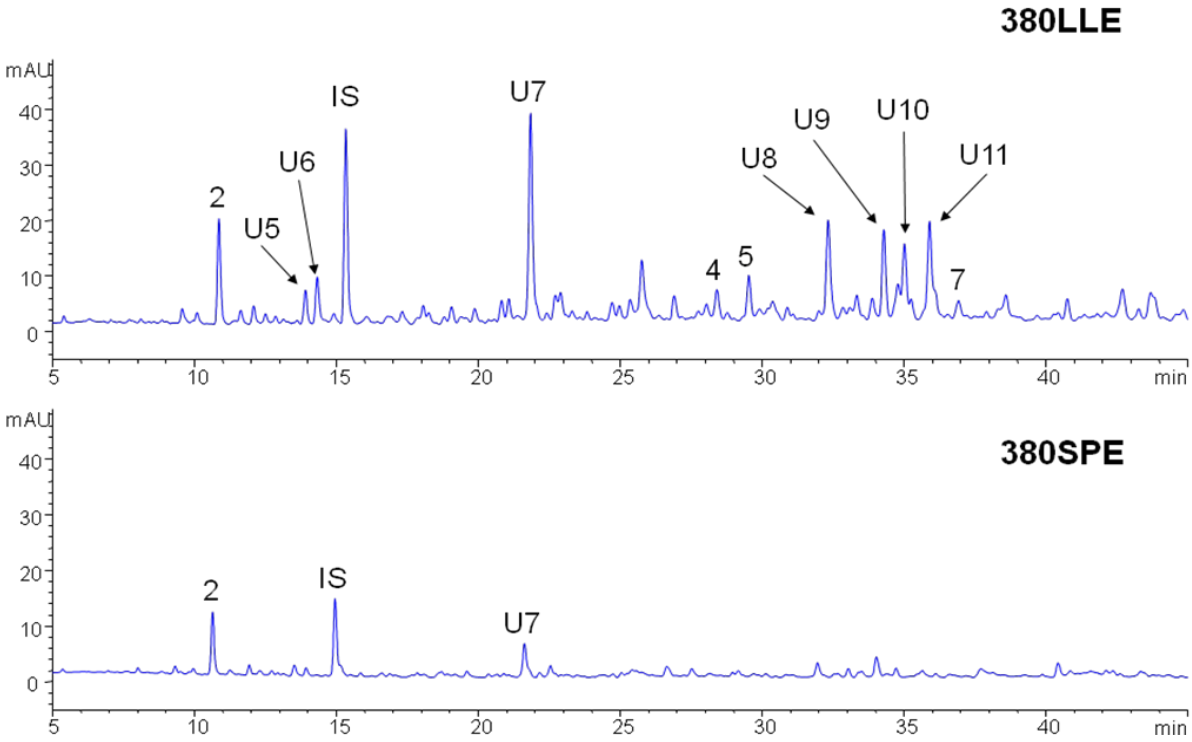

Figure 3: Chromatograms of samples 380LLE and 380SPE maintained at 80° C for 3 months. 2: *p*-HPEA; 4: *p*-HPEA-EDA; 5: Pin + Ac Pin; 7: *p*-HPEA-EA; IS: internal standard. U5-U11: unknown peaks.

From these results it can be assumed that the diolic stationary phase of SPE columns interacts with oxidized phenols, retains them and, at the same time, causes partial retention of the internal standard (Figs. 2 and 3). This interaction would lead to a further non-selective retention of non-oxidized phenolic compounds, retained in column by the presence of oxidized polyphenols linked to the stationary phase. Such a non-selective retention could explain the decrease of the phenolic compounds total area in extracts incubated at 50° for 3 months and the maintenance of the amount of single compounds in relation to the internal standard (Table 1).

Regarding the naturally oxidized samples, the TPAR values shown in Table 3 provided very interesting results.

Table 3

Table 3

		Total peak area	TPAR
A	ALLE	8839.35	0.17
	ASPE	1537.21	
B	BLLE	10811.64	0.28
	BSPE	3019.09	
C	CLLE	36888.21	0.77
	CSPE	28363.43	
D	DLLE	9429.10	0.71
	DSPE	6736.54	

Naturally oxidized samples. A: supermarket sample stored 31 months with a stopper at room temperature and with high headspace; B: D.O.P. Chianti Classico stored 19 months at light conditions and without a stopper to permit photo-oxidation and allow the continuous presence of oxygen. C: Sample produced by Nostrana di Brisighella cv. and stored for 7 months) with a stopper at 9°C; D: LLE extract of sample B subsequently loaded in an SPE column. TPAR is the Total Peak Area Ratio obtained from SPE/LLE values of Total Peak Area of samples subjected to the same storage conditions.

The lowest value was that of sample A, which corresponded to the supermarket virgin olive oil produced in 2003. Thirty-one months of conservation of this low quality, but high priced oil probably led to a strong oxidation of phenols. Thus, the effect of retention in SPE column was high and the TPAR value was comparable to the sample maintained at 80°C for 3 months. In spite of the longer storage time of sample A, the absolute oxidation conditions for samples A and B led to similar oxidation levels in both samples at the end of the storage periods, as reflected in the OFA and PV (Table 4). For this reason, similar TPAR values were expected for samples A and B.

Table 4

Sample	OFA (%)	R.S.D.	PV (meq O_2/Kg)	R.S.D.
A	4.44 ± 0.33	7.38	61.73 ± 1.21	1.97
B	5.57 ± 0.07	1.21	91.37 ± 0.34	0.37
C	1.17 ± 0.07	6.33	29.28 ± 1.40	4.77
0	na	-	7.18 ± 0.06	0.80
350	na	-	29.58 ± 0.15	0.50
380	na	-	85.41 ± 1.39	1.63

OFA: oxidized fatty acids (average ± standard deviation); R.S.D.: relative standard deviation; PV: Peroxide value (average ± standard deviation); na, not analyzed.

Sample C was, however, a very high quality virgin olive oil, produced in 2005 and stored after production in the dark at 9°C. Under these conditions, oxidation of the oil was considerably reduced. Moreover, this sample had high content of phenolic compounds at the beginning of the storage (as evidenced the high pungent and bitter sensory scores) that preserved the oil from oxidative processes. For this sample TPAR showed a value of 0.77. This value was notably higher than those of the two previous samples and more similar to the value obtained for the fresh oil analysed in the first part of the experimental section (1.24). In fact, the TPAR value of 0.77 could be explained by the low (but significant in comparison with a fresh oil) state of oxidation of sample C at the end of the storage. The OFA value of 1.17% in a virgin olive oil with a high phenolic content (Table 4) could also imply partial oxidation of phenolic compounds during storage. Moreover, this OFA value is in agreement with the peroxide value associated with this sample (Table 4). In fact, while samples A and B showed peroxides values of 61.73 and 91.37 meq O_2/kg after strong oxidation, respectively, indicating a high content of peroxidic compounds (the limit for an extra-virgin olive oil is 20 meq O_2/kg), sample C showed a value of 29.28 meq O_2/kg; which showed an oxidation state that can justify the low presence of oxidized fatty acids as shown by the OFA value (Table 4).

From the analysis of extract D further considerations on the potential retention effect by SPE columns can be made. For this extract, the TPAR value was of 0.71, a value obtained from the TPAR between LLE of sample B subsequently loaded in an SPE column in order to eliminate the retention effect of the matrix and the LLE of the same sample. This value was significantly higher with respect to the TPAR of sample B and could be explained by the absence of matrix interference and discarded washing solution. Therefore, the matrix could pose a steric impediment for the elution of some potentially interesting compounds. Together with the previous considerations on retention effect by the stationary phase of SPE columns, this may help explain the complex processes that cause a loss of oxidized and non-oxidized phenolic compounds with the SPE method.

6.5 Discussion

The results with different samples under various oxidation conditions indicate that polar oxidation products (from phenols or lipids) may interfere with the retention of phenols in SPE columns when oxidation is significant. Solid-phase extraction seems to be an effective alternative for fresh samples; however, it could be inappropriate for samples in which oxidation processes have promoted the formation of oxidized phenolic compounds and other oxidation products derived from fatty acids or TAGs. In this work the analysis has been developed in samples subjected to both accelerated and natural oxidation. The total peak area ratio (TPAR) proposed herein showed values close to 1 for fresh samples of virgin olive oil (Table 2). This ratio appeared to decrease rapidly in samples with an increasing content of oxidized polyphenols, and thus a greater reliability of LLE in samples containing oxidized phenolic compounds was observed (Tables 2 and 3). It may be feasible, therefore, to use the ratio fresh phenols/oxidized phenols as an interesting means of determining the freshness/aging of the oil. Additional experimental data on this particular topic may be warranted to confirm the present results.

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7 PROTEINS AND PROTEOLYSIS IN PRE-TERM AND FULL-TERM HUMAN MILK SAMPLES

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Summary statement: The protein fraction of human milk from mothers who give birth to pre-term or full-term babies has not been explored in detail yet. This study provides data on protein content, plasmin activity and a detailed proteomic profile of pre-term and full-term milk samples.

Keywords: preterm, human milk, proteolysis, two-dimensional electrophoresis, proteomics

7.1 Abstract

The protein fractions of human milk samples from mothers giving birth at term and pre-term were

compared through the analysis of total protein content, plasmin activity and mono- (1D) and two-dimensional (2D) electrophoresis. Comparison of total protein content and plasmin activity showed statistically significant differences ($p < 0.001$) between the two groups of samples, with pre-term milk having a higher protein concentration (1.80 % vs 1.09 %) and higher plasmin activity (0.147-0.397 AMC/min vs 0.045-0.104 AMC/min) than term milk. Comparison of 2-D maps showed higher numbers of specific, over-expressed and total protein spots for pre-term milk. The 50 most abundant proteins in term and pre-term milk were identified by mass spectrometry. The specific additional spots were mostly of low molecular weight, i.e., casein break-down products due to the higher plasmin activity. The higher proteolytic activity in pre-term milk may aid the digestion of breast milk in premature babies.

7.2 Introduction

Human milk has long been recognized as the optimum form of nutrition for the newborn period [1]. Human milk contains a wide array of proteins that contribute to its unique qualities and which

provide biological activities, ranging from antimicrobial effects to immunostimulatory functions. In addition, they provide adequate amounts of essential amino acids to growing infants [2].

In milk from most mammalian species, casein is the principal class of proteins, but this is not the case for human milk. In fact, colostrum and "preterm" milk contain little or no casein. With increasing duration of lactation after birth, casein constitutes a progressively larger proportion of human milk protein [3-4]. Caseins in human milk comprise 10-50% of total protein, and whey proteins account for the remainder [3]. β -Casein, the major casein of human milk [5], has a molecular weight of about 24 kDa and consists of 212 amino acids; several potential sites for phosphorylation have been found, especially at the N-terminal [6-7]. Of the whey proteins, human α -lactalbumin has a very high nutritional value and its amino acid composition appears to be very similar to the estimated amino acid requirement of newborns [8]; it constitutes 10-20% of total protein, has a molecular weight of 14.1 kDa and consists of a single polypeptide chain of 123 amino acids [9]. Human milk does not contain β-lactoglobulin, and no α_{s2}-casein has been shown to be present in human milk.

In contrast to processed cow's milk, human milk is a "live" secretion, containing active enzymes, hormones and essential nutrients, presumably in the proper forms and proportions for the infant. In particular, since there are higher levels of proteolytic enzymes in human than in bovine milk [10]. Storrs and Hull [11] suggested that these enzymes may provide the breast-fed infant with significant digestive assistance immediately after birth [12]. The main human proteolytic enzyme, plasmin, consists of two polypeptide chains connected by disulphide bridges. It is formed from its proenzyme, plasminogen, a single-chain molecule, by limited proteolysis at two sites on the polypeptide chain. Plasmin is involved in the hydrolysis of proteins of human milk [13].

This communication reports a detailed biochemical comparison of two sets of human milk samples with the aim of investigating differences in total protein content and plasmin activity between samples coming from mothers who gave birth to premature or mature babies. Characterization of the typical human milk proteome and a proteomic comparison of pre-term and term milk was

carried out using two-dimensional electrophoresis followed by mass spectrometry.

7.3 Material and methods

7.3.1 IRB statement

Ethical approval for the study was granted by the Cork University Hospitals Research Ethics Committee, and consent was subsequently obtained on this basis from all participants in the study.

7.3.2 Collection of human milk samples

Milk samples were collected at the Erinville Hospital, Cork, Ireland, between June 2004 and March 2006. Samples were frozen at -80°C and thawed at refrigeration temperature overnight before the analyses. Seven "term milk" samples were collected after the colostrum production period from lactating mothers with "full-term" infants born after 38 weeks gestation and seven "preterm milk" samples were collected from mothers with infants born between 30 and 37 completed weeks of pregnancy.

7.3.3 Determination of nitrogen content and plasmin activity

The total nitrogen content of human milk samples was evaluated by the Kjeldahl method. Plasmin activity in milk was measured using the method of Richardson and Pearce [14]. This assay uses the non-fluorescent substrate N-succinyl-L-alanyl-L-phenyalanyl-L-lysyl-7-amido-4-methyl coumarin (coumarin peptide), which is hydrolysed by plasmin to give the fluorescent product, 7-amido-4-methyl-coumarin; the rate of increase in fluorescence intensity is proportional to the activity of plasmin present.

7.3.4 Mono- and two-dimensional electrophoresis

The human milk proteins were separated on a 12.5 % acrylamide gel according to the method of Laemmli [15]. The gels were stained with Coomassie Blue (R250) and were digitalized at 300 dpi with a GS-800 densitometer (Bio-Rad, Hercules, CA).

Analytical 2D gel electrophoresis was carried out on 7.5 μL of human milk (~100 μg of protein)

using 7-cm strips, a linear pH gradient from 3-10 (Bio-Rad), and preparative 2D gel analysis was carried on 25 μL of human milk (~350 μg of protein) using 17-cm strips and linear pH gradient 3-10 (Bio-Rad). Human milk was mixed with solubilization buffer (9 M urea, 4% CHAPS, 0.05% Triton X100 and 65 mM DTT). Subsequently, 7 or 17-cm strips (with a linear pH gradient from 3 to 10) were rehydrated in the above solution. Isoelectric focusing was carried out using the Protean IEF Cell (Bio-Rad, Hercules, CA, USA) system until 20,000 kV for 7-cm strips and until 100,000 kV for 17-cm strips. Before the second dimension, the strips were reduced (50 mM Tris HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS and 130 mM DTT) and alkylated in the same buffer containing 130 mM iodoacetamide instead of DTT. The strips were then embedded using 0.6 %, w/v, low-melt agarose on the top of a 12.5% acrylamide gel. SDS-PAGE was carried out using a Criterion® Dodeca Cell electrophoresis unit (Bio-Rad, Hercules, CA, USA) for 7 cm strips and with a Protean® II xi Cell electrophoresis unit (Bio-Rad, Hercules, CA, USA) for 17 cm strips. Gels were stained using colloidal Coomassie blue [16] and stained gels were digitized at 300 dpi using a GS-800 densitometer (Bio-Rad, Hercules, CA, USA).

7.3.5 Image analysis

Images of mono-dimensional analytical gels were analyzed with Quantity One software (Bio-Rad, Hercules, CA, USA) and gel images of two-dimensional analysis were analyzed with PD Quest software (Bio-Rad, Hercules, CA, USA). All samples were analyzed in triplicate and the replicates were used to provide an averaged gel. All the averaged gels were compared in order to evaluate differences between samples belonging to the same group, and between the two groups (pre-term and term). The same gel regions were selected and compared, and spot volume was determined as percentage of total volume of all spots on respective gels. The complete pattern (including all spots) was used for qualitative and quantitative analysis. Qualitative analysis identified specific spots present only in one group, while quantitative analysis determined spots with over- or under-expression (2-fold difference).

7.3.6 Protein identification by MALDI-TOF-mass spectrometry

Spots of interest were excised from preparative 2D gels of pre-term and term milk and washed sequentially with Milli Q (Millipore, Massachusetts, USA) water, 25 mM ammonium carbonate, 25 mM ammonium carbonate-acetonitrile (1:1) and acetonitrile. The spots were dried using with a Speed-Vac (GMI Inc., Minnesota, USA) and the proteins were subsequently digested with trypsin (12.5 μg/μL in 25 mM ammonium carbonate).

Resulting fragments were extracted twice with 50 μL of acetonitrile / water (1:1, v/v) containing 0.1 % trifluoroacetic acid for 15 min. Pooled supernatants were concentrated using a Speed-Vac to a final volume of ~ 20 μL and peptides were immediately spotted onto the MALDI target. Mass spectra were recorded in the reflector mode on an Axima CFR plus MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). The MASCOT search engine software (Matrix Science, London, UK) was used to search the NCBInr database. The following parameters were used: mass tolerance of 100 ppm, a minimum of four peptides matching to the protein, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine and pyroglutamylation of glutamine as variable modifications, and one missed cleavage allowed.

7.3.7 Protein identification by nano LC-mass spectrometry

Stained protein spots were excised manually, washed, digested with trypsin and extracted using formic acid. Protein digests were analysed using an ion trap mass spectrometer (Esquire HCT plus; Bruker) coupled to a nano-chromatography system (HPLC 1200, Agilent) interfaced with an HPLC-Chip system (Chip Cube, Agilent). MS/MS data were searched against NCBI (National center for Biotechnology information) and MSDB databases using Mascot software.

7.3.8 Statistical analysis of data

Total protein and plasmin activity data were analyzed using SPSS r.11.0.0 statistical software (SPSS Inc., Chicago, IL). Differences at a 5% significance level among means were determined by one-way ANOVA, using Tukey's test. Two-dimensional analysis data were also analyzed by principal

components and classification analysis (Statistica 6.0, Statsoft Inc., Tulsa, OK) to analyse correlations between the analyses and differences between the samples.

7.4 Results

7.4.1 Total protein content determination

The protein content of pre-term and term milk samples (Fig. 1, top panel) differed significantly between the two groups.

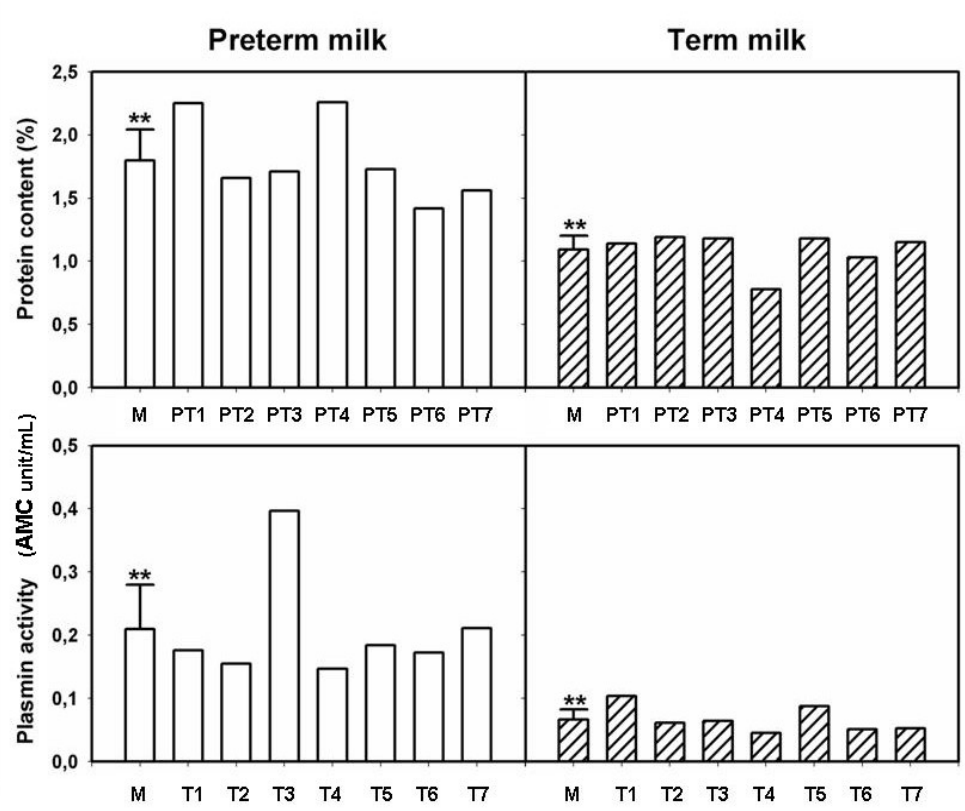

Figure 1: Protein content and plasmin activity of the preterm and term human milk samples; M: average value, PT: preterm, T: term; **: statistically significant differences between the two groups ($p < 0{,}001$).

The mean protein content of the seven preterm milk samples was 1.65 fold higher ($p < 0.001$) than the mean protein content of the seven term milk samples. The mean total protein contents of preterm and term milk samples was 1.8 % and 1.09 % respectively. In addition, the levels of protein in the preterm milk samples were more variable (range of 1.42-2.26%) than the term milk samples (0.78-1.19%).

7.4.2 Plasmin activity

Plasmin activity in preterm and term milk samples were statistically significant (p<0.001) (Fig. 1, bottom panel); the plasmin activity of the seven term milk samples was very consistent and lower (0.045-0.104 AMC units per mL milk) than the plasmin activity of the preterm milk samples (0.147-0.397 AMC units per mL milk).

7.4.3 Selection of the most representative milk samples

Mono-dimensional electrophoresis was performed on 14 milk samples (7 preterm and 7 term; Fig. 2).

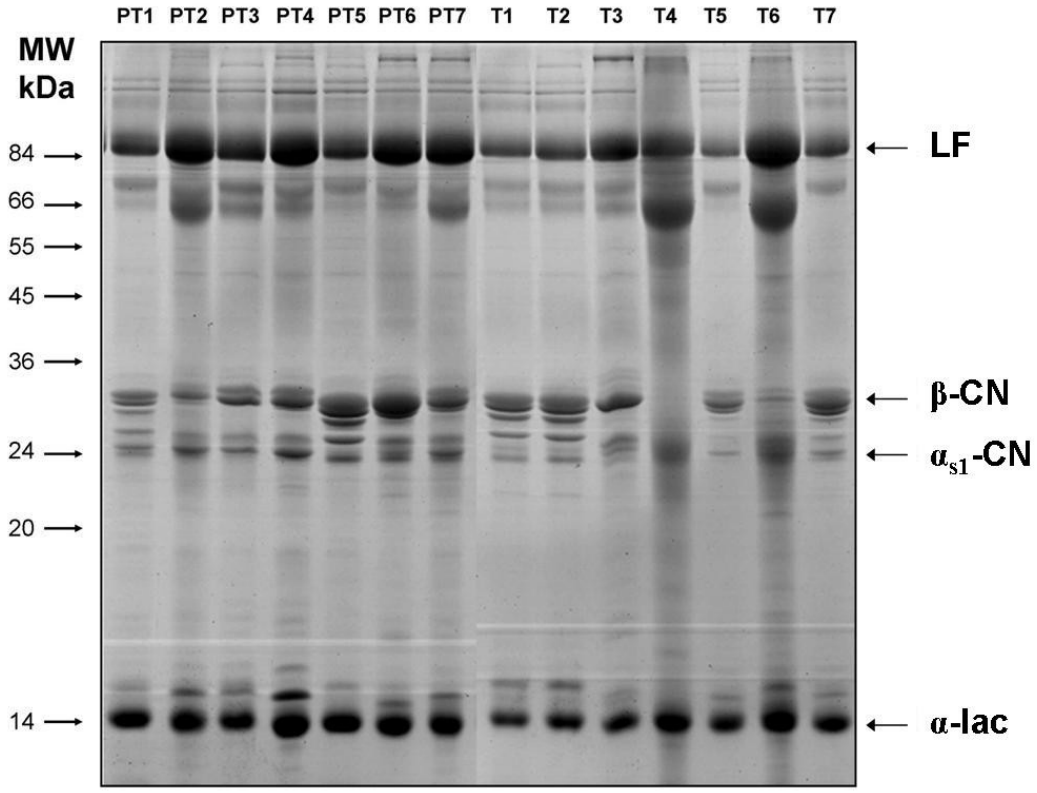

Figure 2: Mono-dimensional SDS-PAGE electrophoresis patterns of 7 milk samples from mothers giving birth at pre-term (PT) and term (T). LF: lactoferrin; β-CN: β-casein; α-CN: α-casein; α-lac: α-lactablumin.

The principal bands in all samples corresponded to lactoferrin, β -casein, α_{s1}-casein and α -lactalbumin. However, there was significant variation in the intensities of the bands corresponding to these proteins between samples, more so in the case of the caseins than the whey proteins. In

addition, several samples showed bands of lower-molecular weight than the caseins (i.e., probably polypeptides produced by proteolysis). The difference between preterm and term samples was less than the inter-sample variability.

Subsequently, to determine a characteristic pattern of the milk samples, a correlation matrix derived from the number and the intensity of the mono-dimensional electrophoretic bands was performed with the Quantity one software. According to this correlation matrix, three most representative samples of each group (PT2, PT4, PT7 for preterm and T1, T2, T7 for term) were chosen and analysed by two-dimensional electrophoresis.

7.4.4 Identification of the most abundant protein spots from human milk: the preterm milk reference map

A two-dimensional electrophoresis reference map of human preterm milk was produced to identify most of the abundant proteins (Fig. 3). Mass spectrometry identifications of the 50 analysed spots are shown in Table 1.

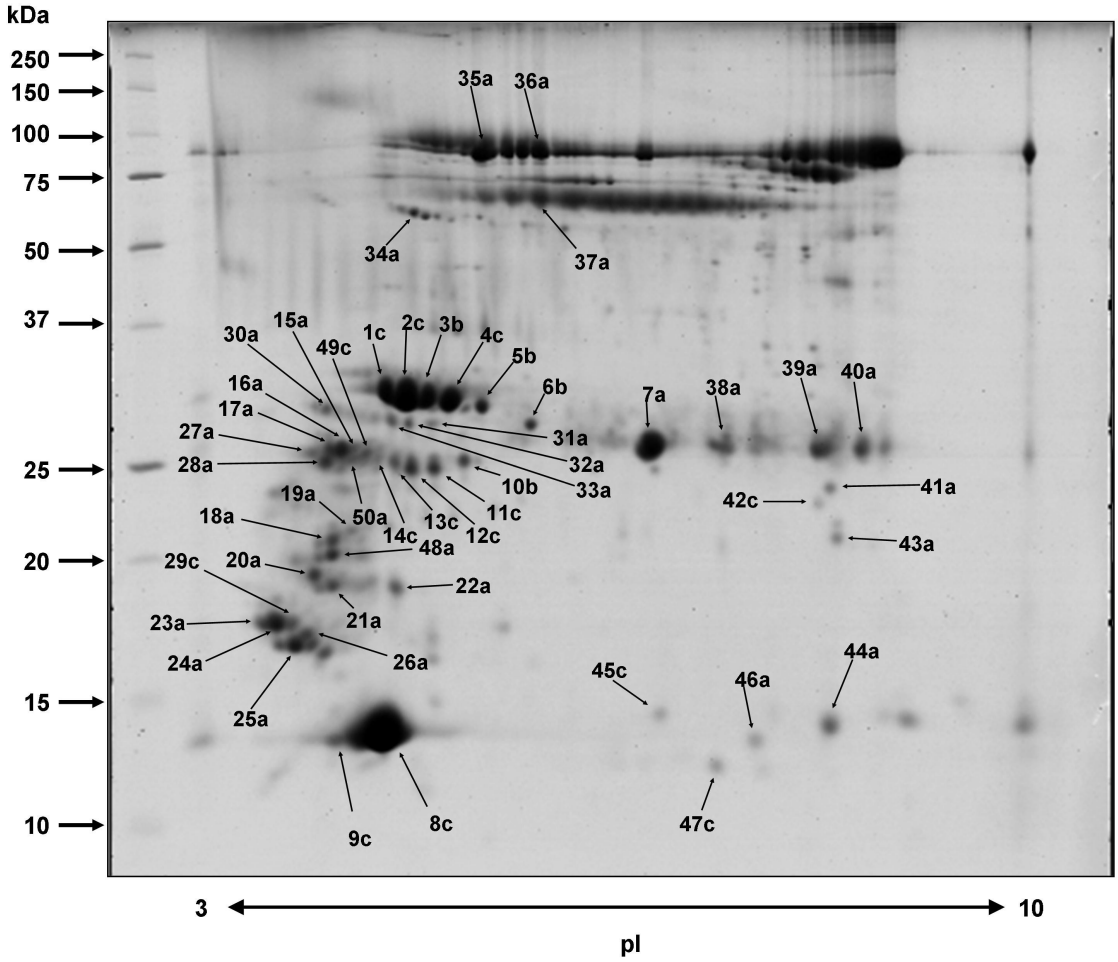

Figure 3: Reference proteomic map of a human preterm milk sample: 2D preparative gel of a representative milk sample (~ 300 µg of proteins) under reducing conditions using a 17 cm pH 3-10 pI range for the first dimension, and a 12% gradient acrylamide gel for the second dimension. The most abundant spots are shown with arrows and were submitted to mass spectrometry identification by MALDI-TOF peptide mass fingerprint and/or nano-LC/MS-MS (results in Table 1). a: over-expressed in pre-term milk; b: over-expressed in term milk; c: no differences between pre-term and term milk.

Table 1: Identification of the 50 most abundant spots from the two-dimensional gel of human milk (see Fig. 3), by peptide mass fingerprint MALDI-TOF. Protein reference (Ref.) corresponds to the Swiss-Prot/NCBI accession number; sequence coverage (% cov.) is given as a percentage. [1]: theoretical molecular weight (MW) and isoelectric point (pI) of proteins according to the amino acid primary sequence and without consideration of any post-translational and/or degradation modifications. [2]: molecular weight (MW) and isoelectric point (pI) as observed with the position of the corresponding spots on the two-dimensional electrophoresis gel.

	Protein name	Ref. Swiss Prot	MW (kDa)		pI		PMF / MALDI-TOF			nanoLC-MS/MS		
Spot N°			theor[1]	obs[2]	theor[1]	Obs[2]	Score	% Cov.	pep	Score	Match pep	Hit rank
1	β-casein	P05814	23.86	35.04	5.33	4.97	71	31	3			
2	β-casein	P05814	23.86	34.78	5.33	5.1	71	31	3			
3	β-casein	P05814	23.86	33.82	5.33	5.23	92	38	4			
4	β-casein	P05814	23.86	34.34	5.33	5.41	71	31	3			
5	β-casein	P05814	23.86	32.49	5.33	5.6	71	31	3			
6	β-casein	P05814	23.86	31.01	5.33	5.81	71	31	3			
7	β-casein	P05814	23.86	27.58	5.33	6.4	92	38	4			
8	α-lactalbumin	P00709	14.08	14.85	4.70	4.97	82	33	5			
9	α-lactalbumin	P00709	14.08	13.57	4.70	5.09				283	8	1
10	α-casein	P47710	20.09	27.36	5.17	5.56	68	22	3			
11	α-casein	P47710	20.09	25.77	5.17	5.32	68	22	3			
12	α-casein	P47710	20.09	25.59	5.17	5.17	70	30	4			
13	α-casein	P47710	20.09	25.77	5.17	5.04	70	30	4			
14	α-casein	P47713	20.09	23.88	5.17	5.24	72	35	5			
15	α-casein	P47710	20.09	26.66	5.17	4.73	70	30	4			
16	α-casein	P47710	20.09	26.58	5.17	4.65	70	30	4			
17	α-casein	P47713	20.09	25.06	5.17	4.75	75	30	4			
18	α_{s1}-casein	P47712	20.09	21.96	5.17	4.77				87	2	4
	β-casein	P05814	23.86	21.96	5.33	4.77				40	3	6
19	α_{s1}-casein	P47711	20.09	22.17	5.17	4.99				102	2	1
	β-casein	P05814	23.86	22.17	5.33	4.99				43	2	2
20	α_{s1}-casein	P47712	20.09	21.6	5.17	4.56				159	3	1
21	α_{s1}-casein	P47713	20.09	21.09	5.17	4.77				98	2	1
22	α_{s1}-casein protease	P47710	20.09	21.28	5.17	5.41	72	35	5			
23	serine	Q6ISJ4	26.7	20	5.72	3.75				61	2	1
24	α_{s1}-casein	P47711	20.09	20.27	5.17	4.03				51	2	1
25	α_{s1}-casein	P47711	20.09	18.88	5.17	4.29				106	2	1
26	α_{s1}-casein	P47713	20.09	18.74	5.17	4.5				72	1	1
27	Ig J chain	P01591	15.59	23.89	4.62	4.53	127	11	59			
28	Ig J chain	P01591	15.59	23.64	4.62	4.7	110	9	59			
29	α_{s1}-casein	P47713	20.09	20.07	5.17	4.23				118	2	1
30	α_{s1}-casein	P47713	20.09	32.05	5.17	4.71				127	4	2
31	α_{s1}-casein	P47713	20.09	28.44	5.17	5.5				213	5	5
	β-casein	P05814	23.86	28.44	5.33	5.5				56	2	13
	Anti-pneumo coccal antibody	Q502W4	25.94	28.44	8.69	5.5				92	3	7
32	α_{s1}-casein	P47713	20.09	28.44	5.17	5.5				301	8	1
	β-casein	P05814	23.86	28.44	5.33	5.5				44	2	3

33	α_{s1}-casein	P47713	20.09	28.61	5.17	5.39				216	5	1
	β-casein	P05814	23.86	28.61	5.33	5.39				74	2	3
	α-lactalbumin	P00709	14.08	28.61	4.70	5.39				121	5	2
34	α 1 antitrypsin	P01009	44.32	69.44	5.37	5.53				799	23	1
	κ-casein	P07498	18.16	69.44	8.68	5.53				51	2	4
35	Lactoferrin	Q2TUW9	77.99	81.24	8.51	5.11	78	12	5			
36	Lactoferrin	Q2TUW9	77.99	83.29	8.51	5.5	94	15	9			
37	Lacto-transferrin	P02788	76.17	71.4	8.47	6.4				263	7	7
	Immunoglobulin	Q9NPP6	44.79	71.4	5.74	6.4				171	4	9
38	Ig k VLG rgion	/	28.6	23.68	6.15	7.31				418	11	1
39	IgG k chain	/	23.3	24	7.75	8.07	140	9	52			
40	IgG k chain	/	23.3	24.95	7.75	8.34	103	6	29			
41	β-casein	P05814	23.86	22.84	5.33	8.28	69	28	3			
42	β-casein	P05816	23.86	22.7	5.33	8.09	69	28	3			
43	β-casein	P05815	23.86	22.08	5.33	8.44	69	28	3			
44	β-casein	P05817	23.86	13.6	5.33	8.31	69	28	3			
45	Fatty acid binding protein	P05413	14	14.73	6.81	6.34				332	9	1
46	β-casein	P05814	23.86	13.12	5.33	7.59				72	2	2
	α-lactalbumin	P00709	14.08	13.12	4.70	7.59				55	2	3
47	β 2 microglobulin	P61769	11.73	12.18	6.07	7.2				160	6	1
48	Lactoferrin	Q2TUW9	77.99	21.65	8.51	4.76				118	2	1
	α_{s1}-casein	P47713	20.09	21.65	5.17	4.76				82	1	2
	β-casein	P05814	23.86	21.65	5.33	4.76				37	4	3
49	α_{s1}-casein	P47710	20.09	26.51	5.17	4.88	75	30	4			
50	α_{s1}-casein	P47710	20.09	25.73	5.17	4.73	72	35	5			

Two thirds of the proteins identified corresponded to α_{s1}- and β-caseins: 24 spots were identified as α_{s1}-casein isoforms with apparent molecular weights from 18.74 to 32.05 kDa and apparent isoelectric points from 4.03 to 5.56; 18 spots were identified as β-casein isoforms (MW= 13.1 to 35.04 kDa, pI = 4.76 to 8.44). Together with these two main groups of caseins, other well known milk proteins were identified, including α-lactalbumin (4 spots), lactoferrin (3 spots), lactotransferrin (1 spot), κ−casein (1 spot) and several immunoglobulins.

Most of these isoforms corresponded to breakdown products or post-translational modifications of the native protein. In case of β-casein, different levels of phosphorylation are well-known and have been reported in previous works [6]. The same consideration can be applied to α_{s1}-casein for which,

in addition to different phosphorylation levels, there were also many break-down products originating from the action of the proteolytic enzymes, producing a number of smaller peptides with different molecular weight and isoelectric point.

7.4.5 Comparison of the 2D-electrophoretic patterns of term and preterm milks

The aim of the present study was to gain a deeper insight into possible molecular responses in human milk for premature babies as well as identifying proteins which are present or up-regulated in preterm milk that may be beneficial for premature babies, as well as improving understanding of protein intake and digestibility by low-birth-weight infants.

Changes in the human milk proteome for the preterm samples as well as term samples were monitored using 2-DE on proteins solubilized directly from the chosen human milk samples. A high resolution 2-DE gel pattern, in a pI range between 3 and 10, was visualised by CBB staining (Fig. 4).

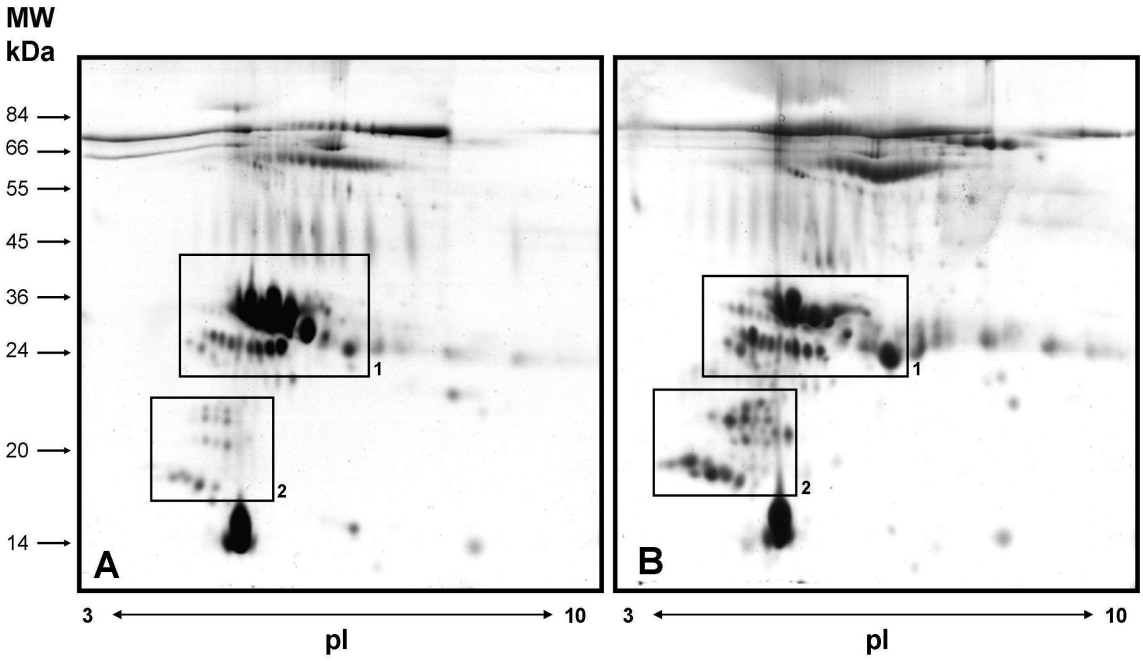

Figure 4: Comparison of preterm and term milk samples: 2D analytical gels of a representative term milk sample (A) and a representative preterm milk sample (B) (~ 100 µg of proteins) under reducing conditions using a 7 cm pH 3-10 pI range for the first dimension, and a 12% gradient acrylamide gel for the second dimension. Two areas of interest were selected in these pictures and were compared in figure 6 for a proposed biological mechanism.

The three replicates selected by mono-dimensional electrophoresis were used for image analysis. A

mean of 101 and 147 protein spots were reproducibly detected in each term and preterm gel, respectively (Table 2) and were selected for further analysis.

Table 2: Number of spots of human milk preterm and term samples and their distribution after 2D-E image analysis using the PD-Quest software.

	Preterm samples	**Term samples**
	X ± SD	**X ± SD**
Sample 1	162 ± 2	92 ± 2
Sample 2	143 ± 4	112 ± 2
Sample 3	136 ± 1	97 ± 1
Mean	147 ± 14	101 ± 10
Common spots	84	
Specific spots	69	10
Over-expressed spots	48	7
Under-expressed spots	7	48
No differences	29	29
Total spots	**153**	**94**

Samples: 1 = PT2 and T1; 2 = PT4 and T2; 3 = PT7 and T7; X = average number of spots among three replicates of the same sample. SD = standard deviation of the number of spots among three replicates of the same sample.

Quantitative analysis, using PD-Quest software, revealed than 55 proteins showed more than 2-fold differences in expression value. Among these, a total of 48 proteins were up-regulated and 7 were down-regulated in preterm milk samples. In addition, 69 new spots appeared in the 2D map of pre-term samples; in comparison, only 10 new spots were detected in term samples. Interestingly, most of the up-regulated and specific spots in preterm milk were located in the lower molecular weight area of the gel. It can thus be hypothesized that such specific low molecular weight spots correspond to fragments of proteins after enzymatic hydrolysis.

The differential protein expression profile between term and preterm milk samples was also investigated through Principal Component Analysis (PCA). The spot data for preterm and term milk samples were used for the statistical analysis. As shown in Figure 5, the PCA was able to separate pre-term from term milk samples only in relation to their 2D protein profiles, showing the robustness of the proteomic comparison strategy.

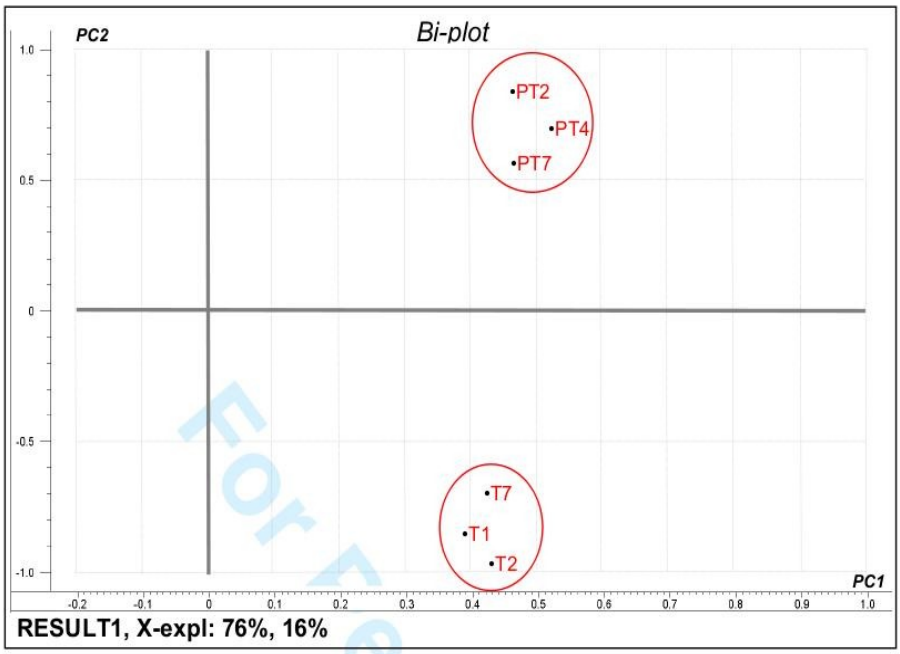

Figure 5: Principal Component Analysis score plot based on two-dimensional electrophoresis analysis.

7.5 Discussion

Comparative proteomics, as well as biochemical and enzymatic measurements, were employed to investigate the modulation of proteins in milk from mothers who gave birth to pre-term and full-term babies.

The total protein content measured was consistent with that reported by Atkinson et al [17], who first showed that the total protein concentration of breast milk from women delivering prematurely was considerably higher than that of milk from women delivering at term, as well as the level of non-protein-nitrogen. It can be hypothesized that a higher protein concentration can benefit the premature infant with its rapid catch-up growth and high protein requirement.

Atkinson et al [17] also reported that the peptides found in human milk may be indigenous constituents of breast milk or may be formed in expressed milk through the proteolytic action of native enzymes in the milk. Building on these findings, this paper reports, for the first time, increased plasmin activity in milk from women delivering prematurely. Milk proteases present in breast milk, including plasmin, are capable of hydrolysing β-casein in milk [7, 13, 18] and could contribute to the complement-inhibitory activity of human milk by non-immune splitting of native

complement components [19]. This study adds new information about the protein composition of human milk, underlining the key role of plasmin: its higher level in milk for premature babies results in higher hydrolysis of protein, leading to the production of small peptides which could make the digestion process easier. The higher activity of plasmin in pre-term milk samples could alternatively be the result of increased passive leakage due to altered structure of the mammary gland resulting from the physiological stress experienced by the mother in premature birth; this may be analogous to similar changes reported for mammalian milk during mastitis [20].

Mono-dimensional electrophoretic data showed a more complex pattern for pre-term milk samples, consistent with the plasmin activity results. Higher plasmin activity and higher protein content thus result in higher rates of hydrolysis of protein, that result in the formation of several lower molecular weight polypeptides and peptides. This is translated on the gel as an increase of spots in the lower area and a decrease in the intensity of the major native caseins (Fig 6). The new specific spots with low molecular weight possibly result from the hydrolysis of caseins.

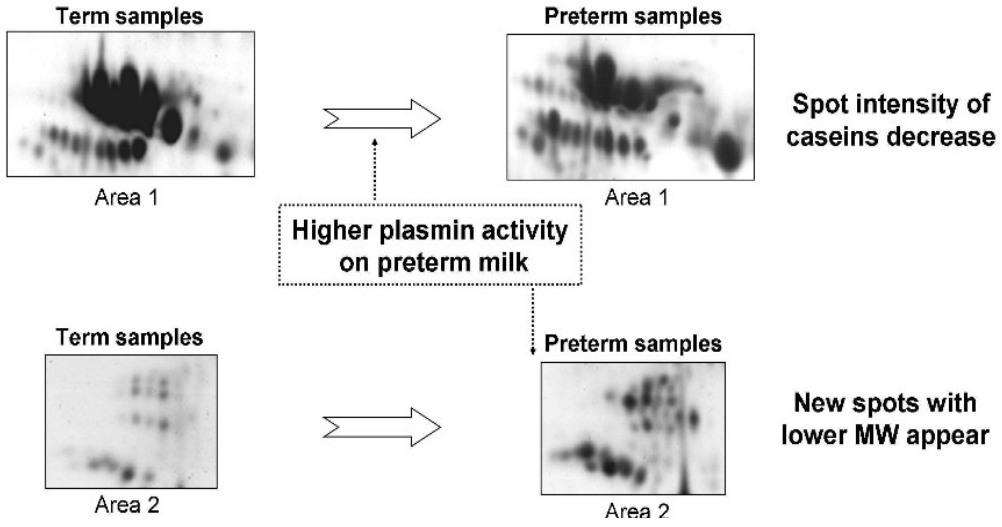

Figure 6: Proposed mechanism for the modulation of proteins behaviour between pre-term and term milks (2D gel localisation of the areas 1 and 2 were shown on the figure 4).

The main protein band of α-lactalbumin showed no particular differences between the two groups, the content of which was similar in all samples, independent of the group to which they belonged. The concentration of α-lactalbumin is linked to the concentration of lactose which is linked with the

osmotic pressure of blood in the mammary gland; this may explain the similar concentration of α-lactalbumin in pre-term and term milk samples (Fig. 2).

In contrast, the casein bands were more intense in the term samples; this could be due to the effect of the higher proteolytic activity of pre-term samples. The increased variety of "pre-digested" proteins in pre-term samples may lead to an easier and more rapid digestion of the protein fraction of breast milk for pre-mature babies (Fig 6).

Moreover, two-dimensional electrophoretic data were further analysed by principal component analysis (PCA) (Fig 5) which explained 92 % of variance in the data, of which 76 % was explained by PC1. Figure 5 shows that this analysis can be used to differentiate between the human milk samples. The preterm and term human milk samples were clearly grouped together (Fig. 5) with two clusters formed by the three preterm milk samples and three term milk samples, indicating more significant differences between these groups than between individual samples.

Identification of the spots of interehttps://www.mymeteor.ie/go/freewebtextst has added to knowledge on the protein fraction of human milk. The first consideration is related to the break-down products, present mainly in the pre-term milk samples, which are mostly poly-peptide fragments derived from α_{s1}- and β-caseins. This observation is complementary to a previous study that reported that β-casein was the main source of small peptides by proteolytic action [13]. In that study, TCA was used to precipitate casein and separate peptides from milk and liquid chromatography-mass spectrometry and tandem mass spectrometry were used for the separation and identification of the peptides. In our study, milk was analysed directly without additional extraction or enrichment steps and the proteins were separated using two-dimensional electrophoresis. These differences in the experimental methods have a great influence on the determination of proteolysis products from proteins. On a first hand, the use of TCA for the purification of peptides and reverse-phase HPLC for the separation gave rise to peptide populations with too low molecular weight to be resolved on a SDS-PAGE gel. On the other hand, two-dimensional electrophoresis allowed us to observe the native proteins preferentially hydrolysed by

plasmin and the resulting polypeptides. To summarise these data, in human preterm milk, plasmin can hydrolysed both α_{s1}-casein and β-casein [13], and yields poly-peptides of both α_{s1}-casein and β-caseins and small peptides of β-casein [13].

Moreover, Ferranti et al [13] reported that, in term milk, the amount of shorter peptides was higher compared with those of greater size which indicates the hydrolysis of casein at higher qualitative and quantitative levels and that α_{s1}-casein-derived peptides, which are few in pre-term milk, were more abundant in term milk. This study, in contrast, reported significantly higher plasmin activity in pre-term milk which results in higher rate of proteolysis. In bovine milk, proteolytic enzymes, and especially plasmin, preferentially hydrolyse β-casein, but in human milk this proteolytic activity is apparently carried out on both α_{s1}-casein and beta-casein.

The quantification data coming from the image analysis highlighted that pre-term milk samples were also richer in immunoglobulins and antibodies, making stronger the hypothesis of milk "naturally enriched" in proteins and peptides but also in bio-active compounds able to protect the pre-term babies during early life.

7.6 Conclusion

This paper reports new information on the protein composition of human milk. The higher total protein content of milk from women delivering prematurely confirms previously reports by other authors and a higher content of immunoglobulins and antibodies in that milk shown. New results related to elevated plasmin activity and its consequences in pre-term milk are presented. Moreover, the proteomic patterns of preterm and term milk samples were investigated in details and the fifty most abundant proteins identified. Our data suggest a physiological response that adjusts enzyme/protein expression to improve milk digestibility for breast-fed prematurely new-born babies.111

7.7 References

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8 CONCLUSIONS

Analytical methods have always been used for the determination of parameters of quality of food products. The use of innovative techniques as HPLC or mono and two-dimensional electrophoresis brought new advantages to this topic. Infact, increased sensitivity and selectivity allowed the determination and quantification of compounds which can be identified as marker of the quality of food products. Moreover, mass spectrometry techniques, as MALDI-TOF or nano LC-mass spectrometry allow to run complete and detailed proteomic studies with the identification of proteins and peptides of interest. These techinques have been shown to be widely helpful for the characterization of particular food matrixes; protein patterns and the understanding of dynamics of proteolysis in food products can also contribute to the evaluation of the quality of food products giving a precious help to the analysis and development of food formulas.

During this PhD project, three analytical methods, strictly related to the evaluation of food quality, were set up and improved. These methods are related to the determination and quantification of phenyl-lactic acid (Armaforte *et al.,* 2006; Coloretti *et al.,* 2007), biogenic amines in low acid salami (Coloretti *et al.,* 2008), and oxidized polyphenols (Armaforte *et al.,* 2007) in olive oil after heat treatment. A detailed proteomic study of human milk, with particular emphasis to the evaluation of differences between human milk for preterm or term babies, have been developed (Armaforte *et al.,* under peer review process); protein patterns and proteolytic dynamics of these two groups of samples have been described bringing new knowledge and possible new inputs for the formulation of specific human milk fortifiers for preterm babies.