Detection of adulterations of food products

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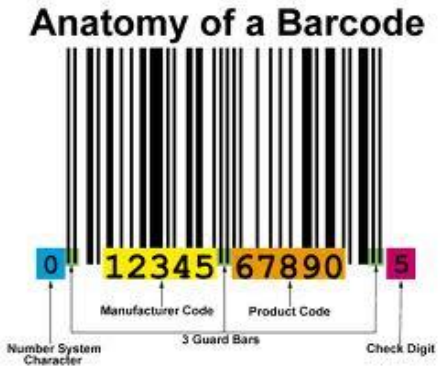


Authenticity of food products

- **Commercial adulteration**

➢ Dairy industry: e.g. replacing sheep's milk with cow's milk in cheese production, addition of melamine

➢ Meat industry: e.g. horse meat in bovine meat products

➢ PDO products (higher commercial value)

- **A number of issues is raised**:

➢ Health (allergies)

➢ Diet (nutritional value & calories)

➢ Religion (absence of pork)

➢ Lifestyle (Vegetarianism & Organic food)

PDO products (Protected Designation of Origin)







- Specific geographical region
- The quality or the characteristics of the product are attributed to the specific regional environment (natural and human factors, climate, quality of the soil, experience)
- The production, processing and preparation of the product takes place within the specific geographical region

Reasons for establishing and protecting PDOs:

❑ Encouragement of the local rural production

❑ Protection of designation from misuse and imitation

❑ Improved understanding of the special character of these products from the consumers

E.C. Regulation 178/2002

- **Traceability** is the ability to trace and follow food, feed, and ingredients through all stages of production, processing and distribution

Methods for identification of species

- **Meat products**:
  - Anatomical
  - Histological
  - Microscopic
  - Organoleptic

  → Conventional

  - Immunological
  - Electrophoretic
  - Chromatographic

  → Analytical

- **Milk & milk products**:
  - Immunological
  - Electrophoretic
  - Chromatographic

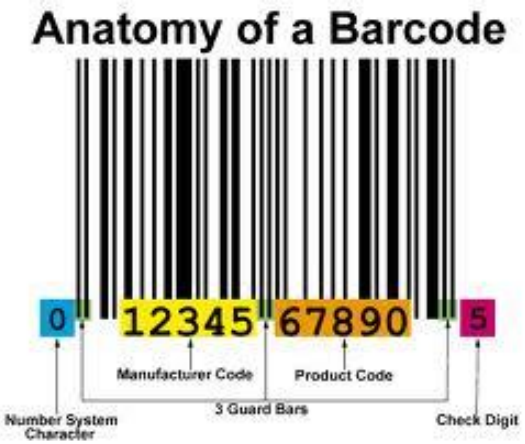
  → Analytical

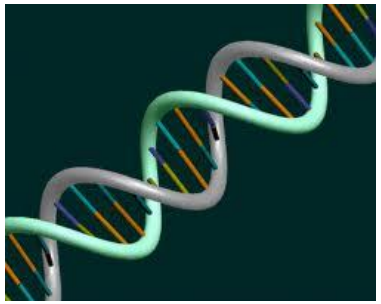
Molecular methods

- DNA based methods of identification
- More reliable (stability of DNA under high temperatures, pressure & chemical treatment)
- PCR (more popular & widely used)
- High sensitivity, reproducibility & simplicity

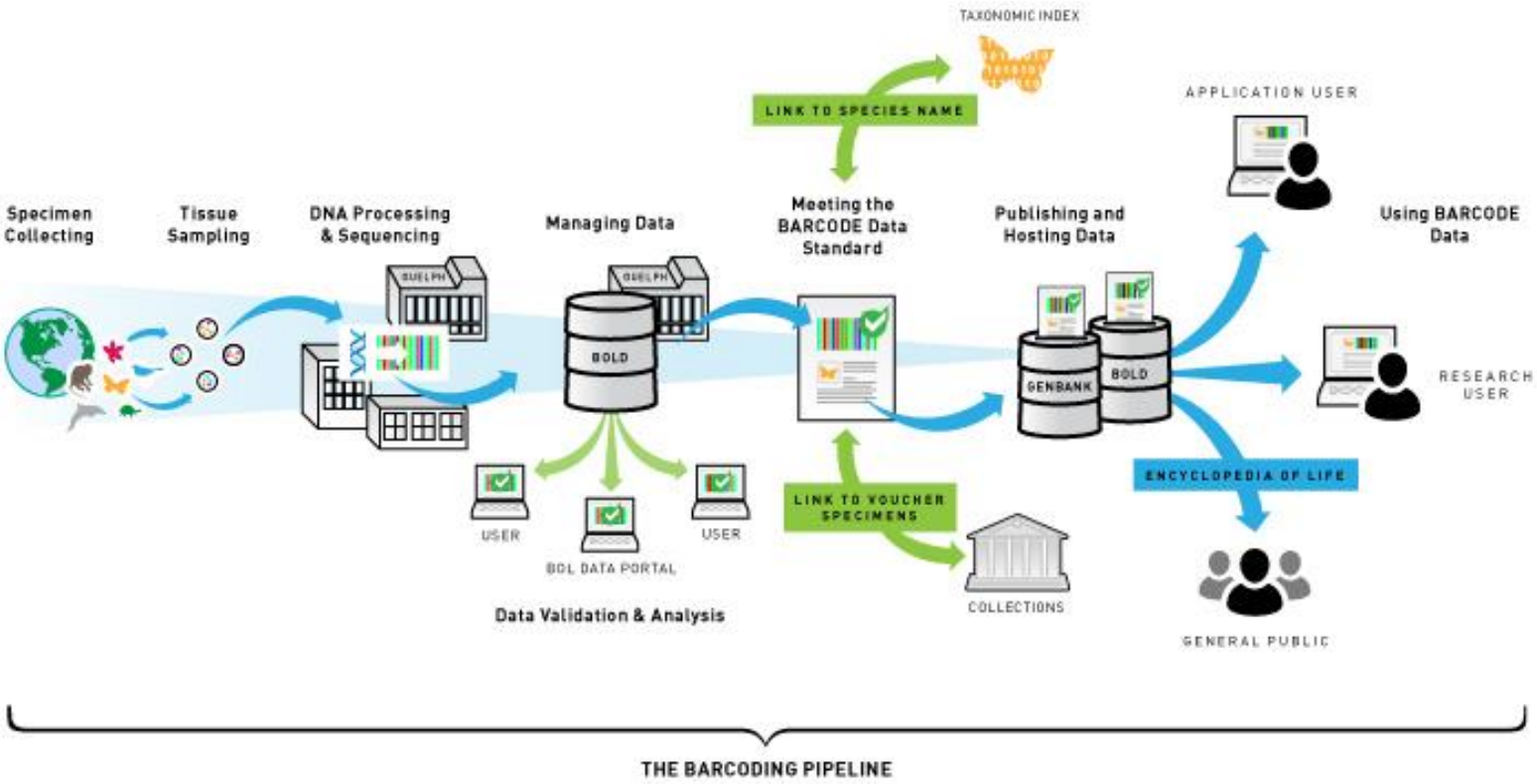
What is DNA barcoding ?

- **DNA barcoding** is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species.





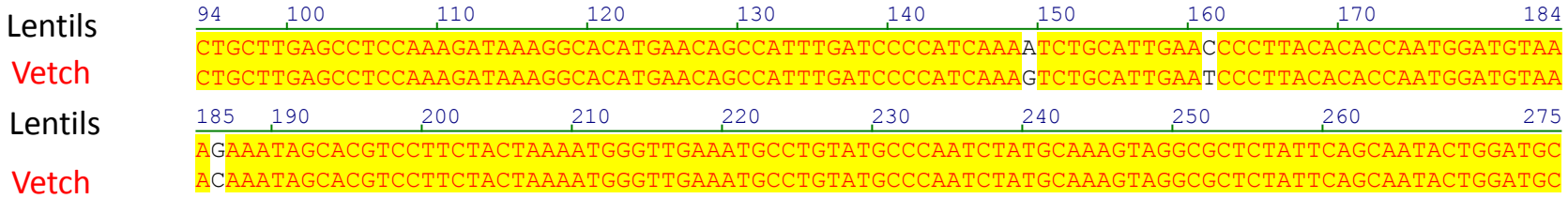
DNA barcoding



In 2003, Paul Hebert, researcher at the University of Guelph in Ontario, Canada, proposed “DNA barcoding” as a way to identify species. Barcoding uses a very short genetic sequence from a standard part of the genome the way a supermarket scanner distinguishes products using the black stripes of the Universal Product Code (UPC). Two items may look very similar to the untrained eye, but in both cases the barcodes are distinct.

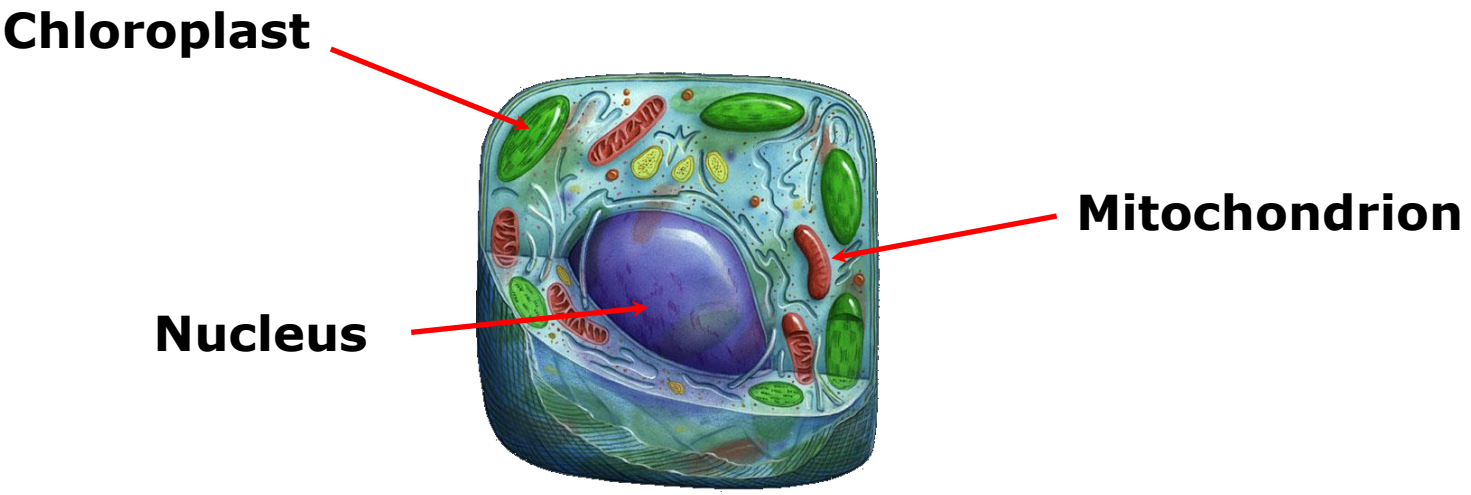
http://www.barcodeoflife.org/content/about/what-dna-barcoding

DNA barcoding



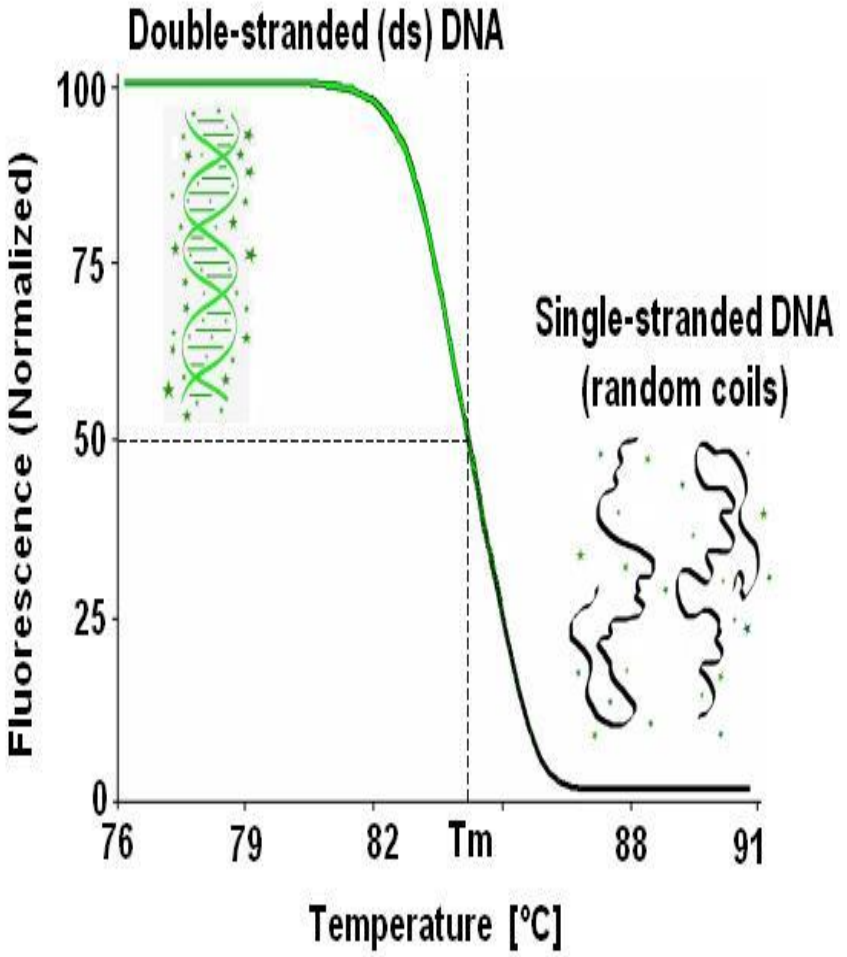
Identification of species

- **DNA molecular markers for barcoding**
  - Chloroplastic (plant cells)
  - Mitochondrial (animal cells)
  - Genomic



HRM-High resolution melting analysis

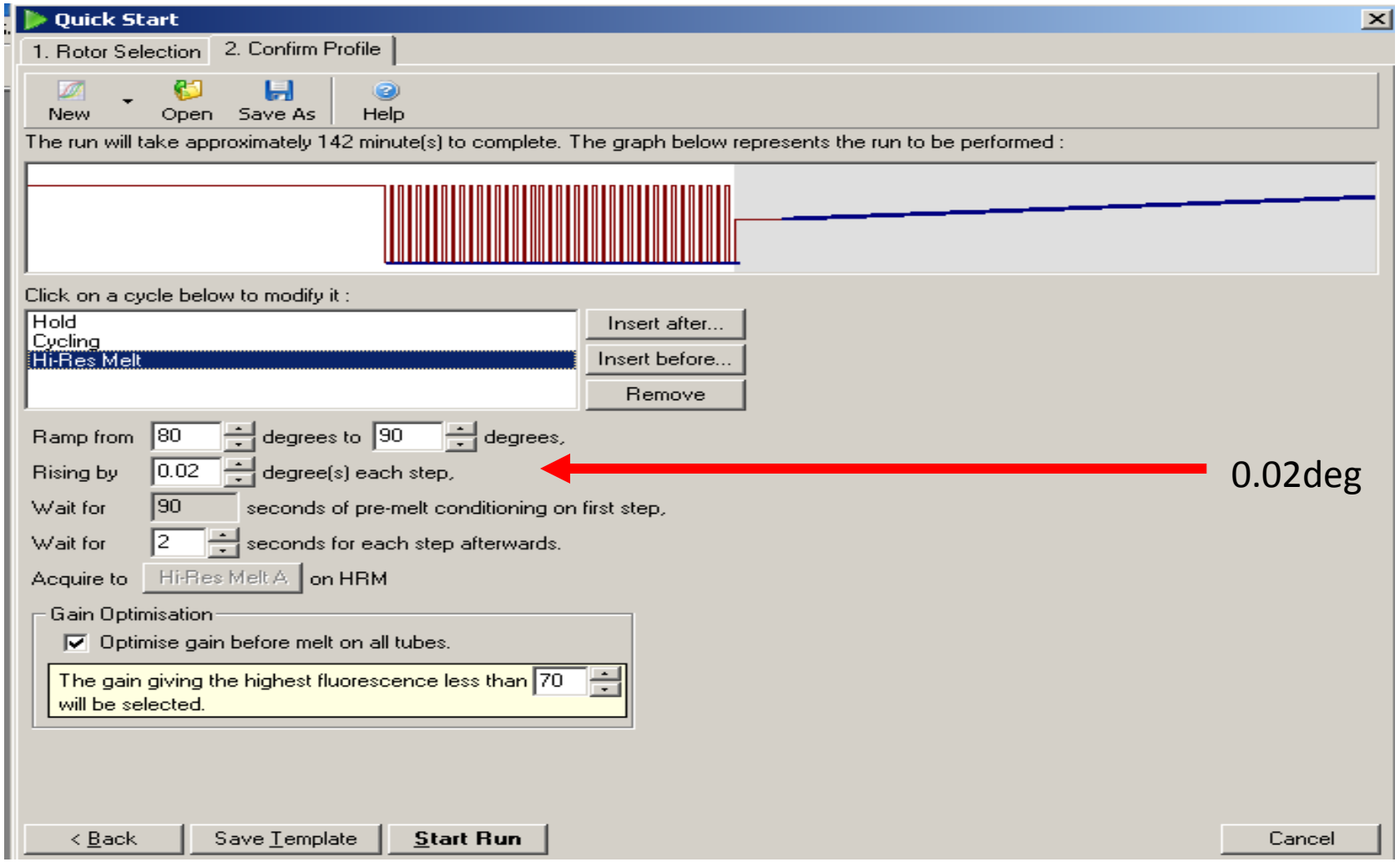
- Post PCR analysis method for identifying genetic variation in nucleic acid sequences
- It measures the melting temperature of an amplified PCR product
- Monitoring of the decrease of fluorescence related to the dissociation of double stranded DNA
- Produces melting curves, unique for each product
- Melting curves are dependent from the composition of the DNA sequence, length, GC content & strand complementarity.



Applications of HRM

- Detection of Single Nucleotide Polymorphisms (SNP)
- Detection of DNA methylation
- Detection of unknown mutations
- Analysis of Microsatellites
- Mapping of genome
- Identification of species
- Quantitative detection of adulteration in food products.

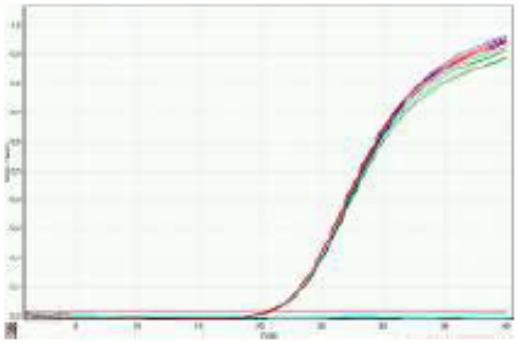
HRM


Quick Start
1. Rotor Selection
2. Confirm Profile
New
Open
Save As
Help
The run will take approximately 142 minute(s) to complete. The graph below represents the run to be performed :
Click on a cycle below to modify it :
Hold
Cycling
Hi-Res Melt
Insert after...
Insert before...
Remove
Ramp from 80 degrees to 90 degrees,
Rising by 0.02 degree(s) each step,
Wait for 90 seconds of pre-melt conditioning on first step,
Wait for 2 seconds for each step afterwards.
Acquire to Hi-Res Melt A on HRM
Gain Optimisation
Optimise gain before melt on all tubes.
The gain giving the highest fluorescence less than 70 will be selected.
< Back
Save Template
Start Run
Cancel

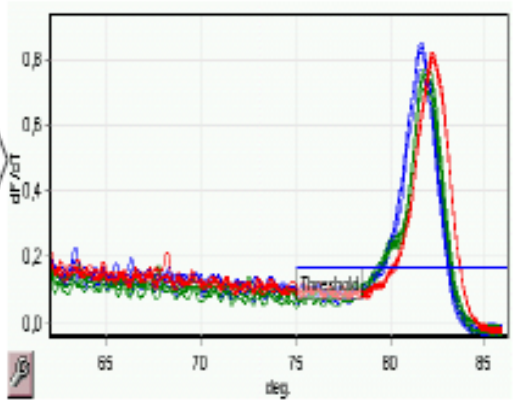
0.02deg

HRM- From PCR to the final result

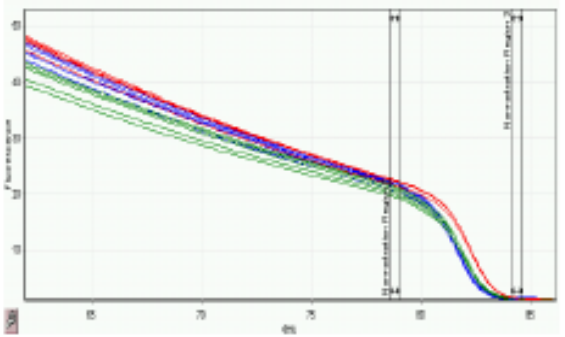
Step 1: Amplification
Was the amplification successful?



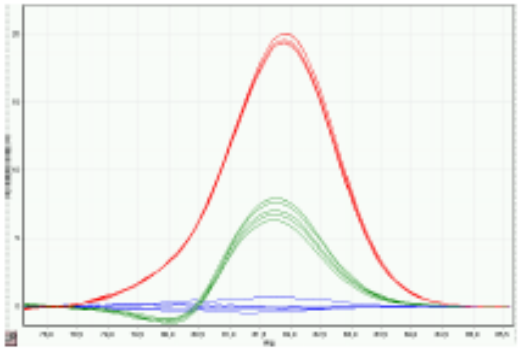
Step 2: Melt curve analysis
Check to verify amplification specificity

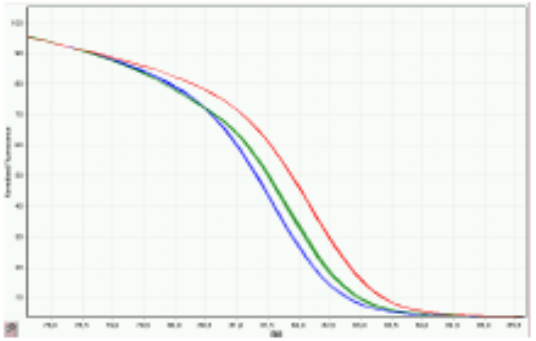


Step 3: Normalisation
Select suitable samples and analysis range



Step 4: Difference plot
Select the reference genotype

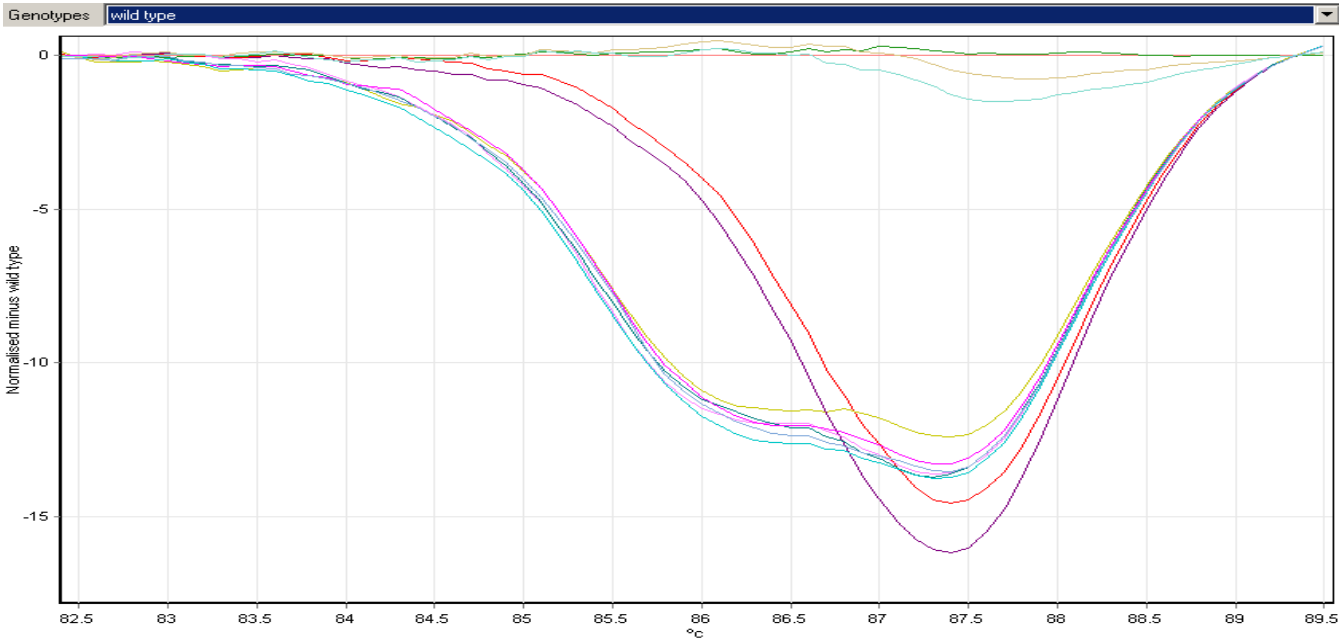




Step 5: Autocalling genotypes
unknowns will be either related to known genotypes or will be marked as variation

SNP Klasse 1		SNP11
Name	Genotype	Confidence %
TT SNP 11 WT	WT	100
Unknown	WT	99.83
Unknown	WT	99.85
Unknown	WT	99.82
Unknown	WT	99.97
TT SNP 11 Hetero	Hetero	100
Unknown	Hetero	99.97
Unknown	Hetero	96.82
Unknown	Hetero	99.02
Unknown	Hetero	99.76
TT SNP 11 Mut.	Mut	100
Unknown	Mut	99.7
Unknown	Mut	99.74
Unknown	Mut	99.97
Unknown	Mut	99.71

Difference Graphs



- Difference graph displays the difference between each sample and a given genotype control
- Allows a calculated percentage confidence relative to a known genotype

Some Examples

- Certifying products made from specific plant or animal species or races using DNA Barcoding techniques
  - Unique signature to be used in all the production chain – traceability
  - Protect the consumers from fraudulent products
  - Maintain the added value of traditional products
  - Protect investments made to develop new food products
- **Obtained Results:** Certification of Feta cheese, Mozzarela di Buffala Campana, Buffalo meat products, Hammon from Greek black pig, etc.

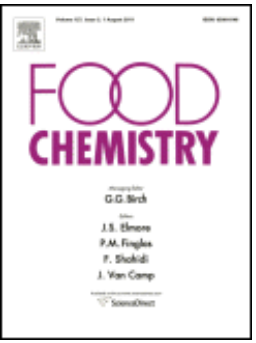
Feta Cheese

+ Only **sheep** and **goat** milk

+ **Goat** milk up to **30%**

- Forbidden production of feta from other kind of milk

2013

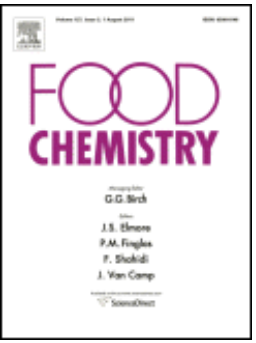


Feta Cheese

Material and Methods

- RT-PCR using bovine d-loop primer pair for detection of bovine milk
- Duplex PCR with HRM analysis using ovine tRNA primer pair and caprine d-loop primer pair for quantifying the ratio of sheep / goat milk

2013

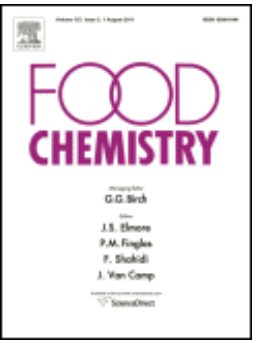


Feta Cheese

Results

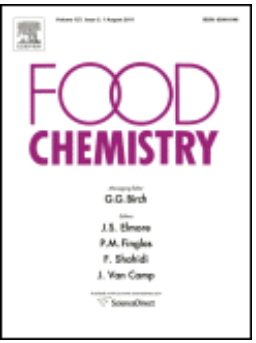
- Limit of detection (**LOD**) was **0.1%** addition of bovine milk in the mixture of sheep/ goat milk

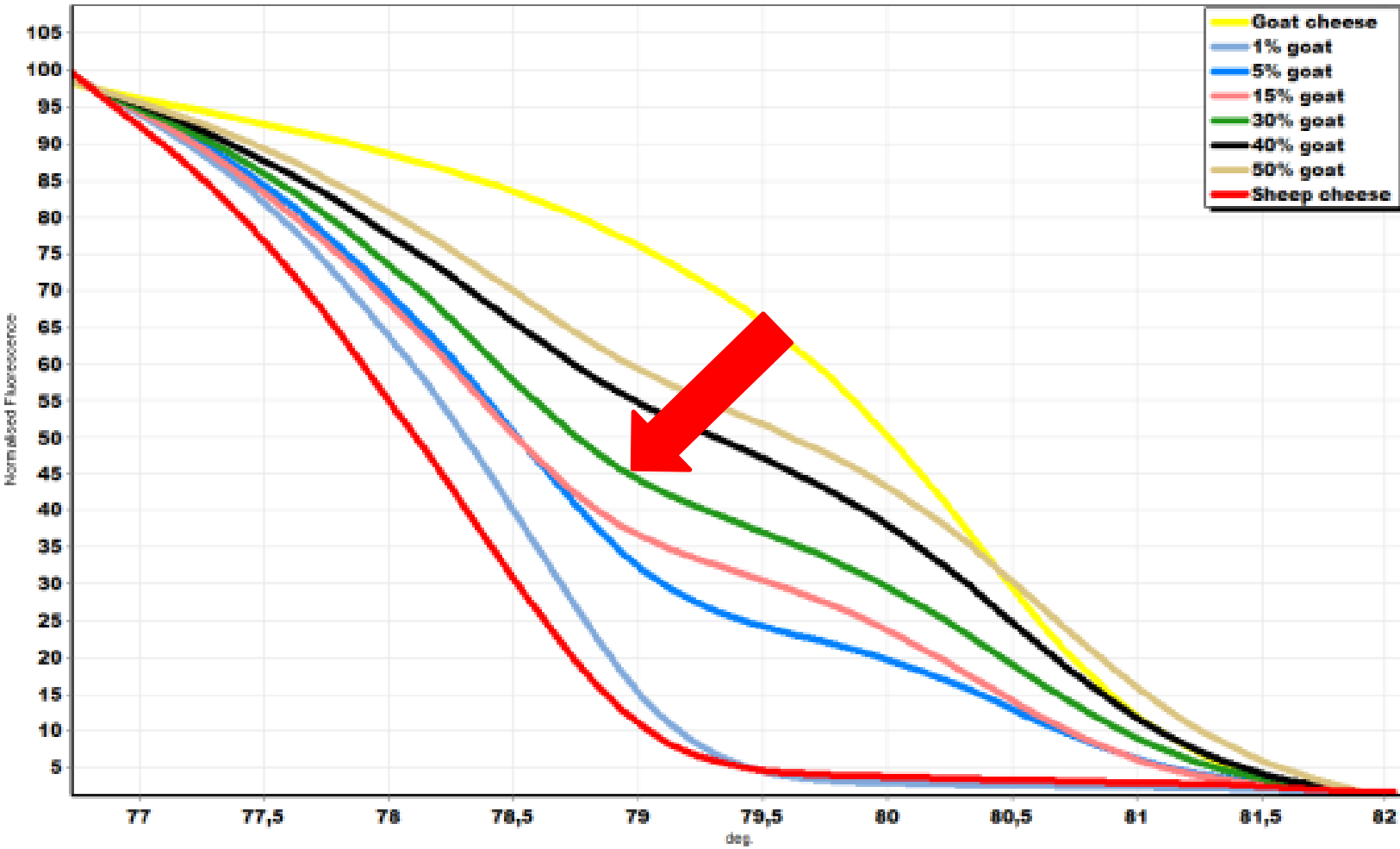
2013



Feta Cheese

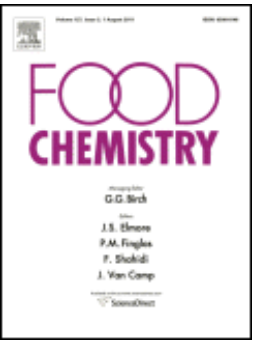
2013

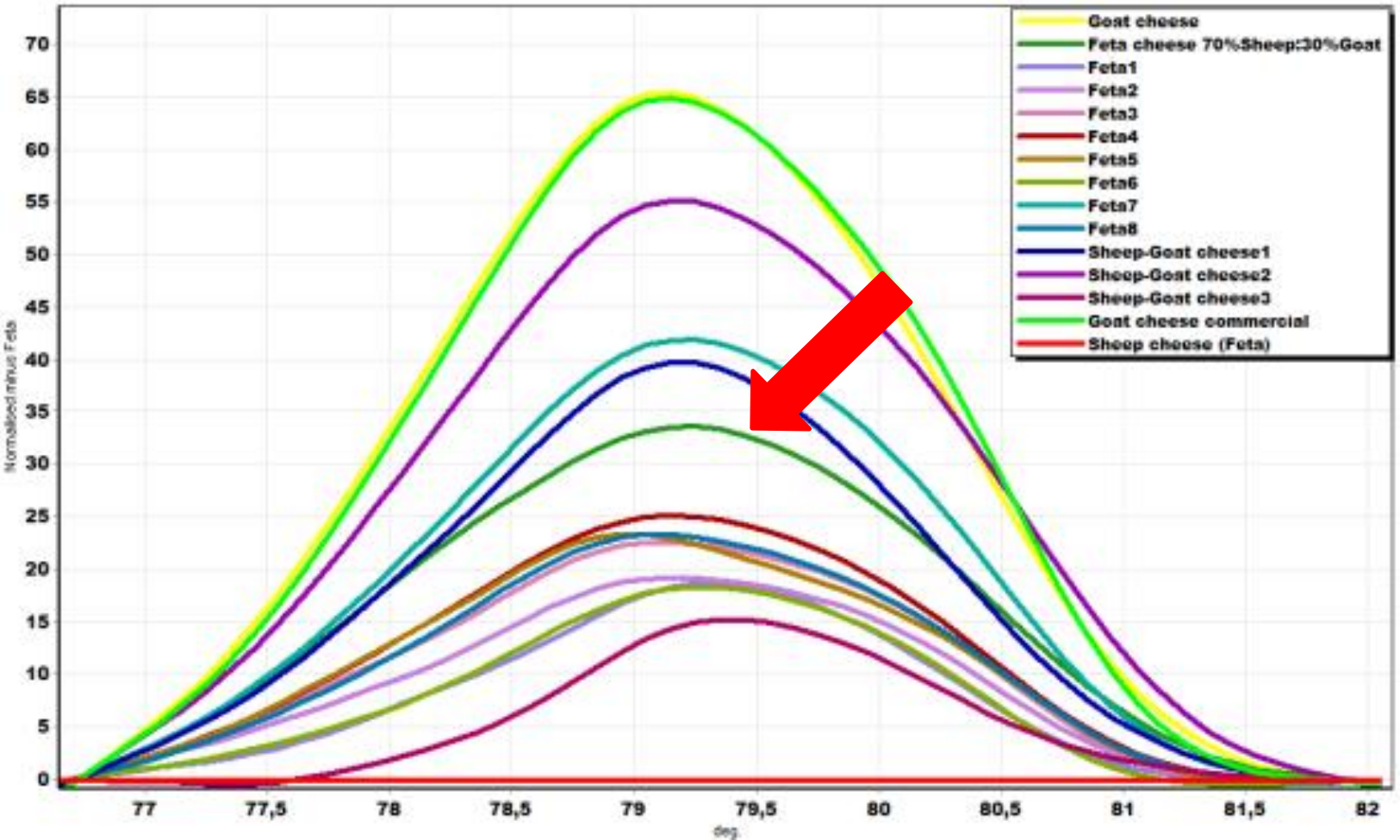




Feta Cheese

2013





Mozzarella di Bufalla Campana and other buffalo dairy products

+ Only **Buffalo** milk

- The addition of bovine or other kind of milk is forbidden

2013

Mozzarella di Bufalla Campana and other buffalo dairy products

- 12S rRNA specific buffalo primer pair
- D-loop specific bovine primer pair
- alternative specific 12S rRNA bovine primer

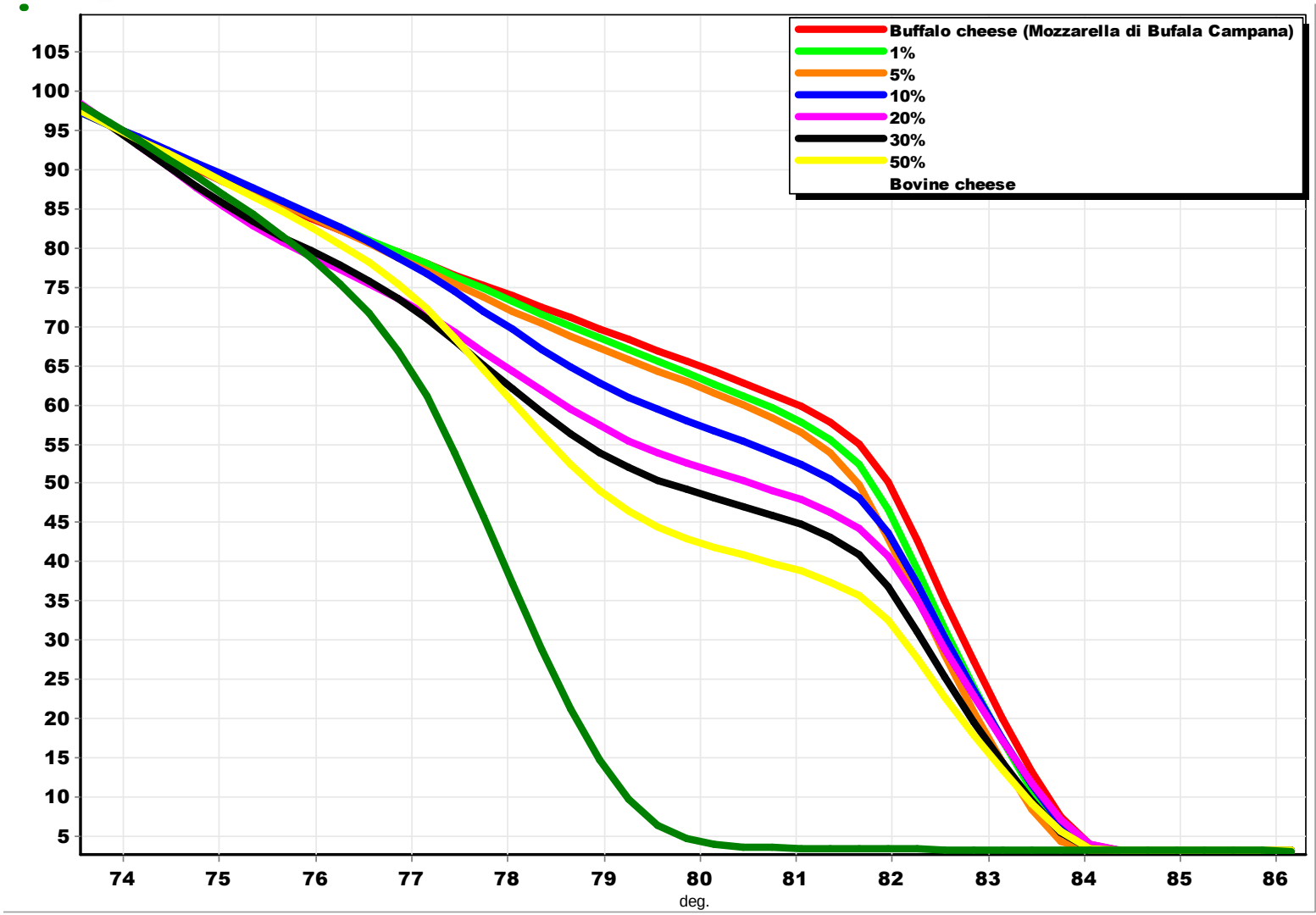
2013

Mozzarella di Bufalla Campana and other buffalo dairy products

Buffalo dairy product	Buffalo specific 12S	Bovine specific D-Loop	Bovine specific 12S
Mozzarellla di Bufala Campana	+	-	-
Burro di Buffala	+	+	+
Buffalo butter	+	-	-
Buffalo cream	+	-	-
Buffalo yogurt	+	+	+
Buffalo rice pudding	+	-	-
Kazan dipi	+	-	-
Buffalo milk	+	-	-
Buffalo ariani	+	-	-

2013

Mozzarella di Bufalla Campana and other buffalo dairy products



2013

Buffalo meat products

2013

- **Buffalo meat** frequently implicated in **meat adulterations**

- **Europe & Egypt**: Adulteration of **buffalo** meat with **bovine** meat

- **India**: Adulteration of **buffalo** meat with **bovine** meat & adulteration of **goat** and **sheep** meat with **buffalo** meat

Buffalo meat products

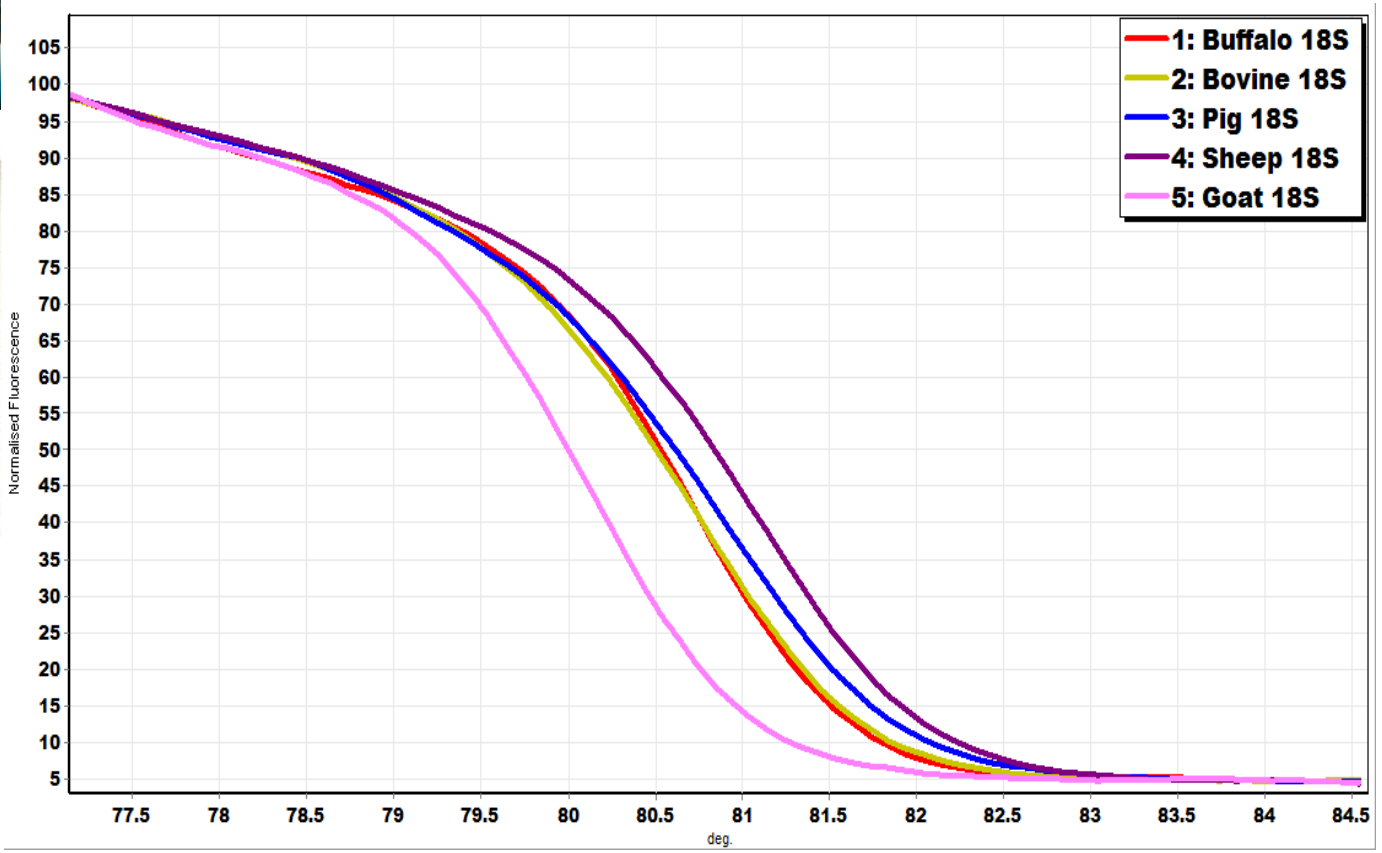
2013

Primers selection

- 12s rRNA buffalo specific primer pair
- 18s rRNA universal for animal DNA

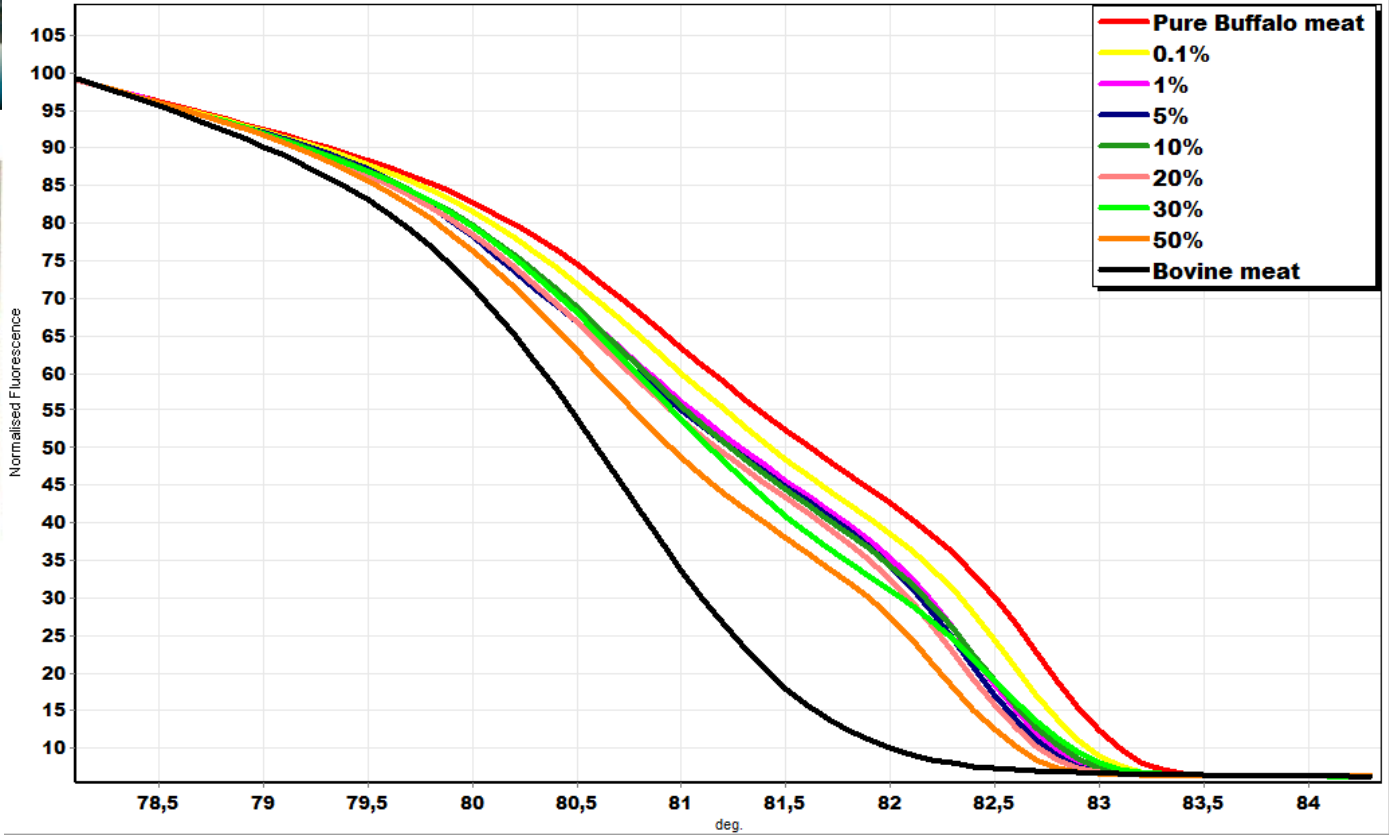
Buffalo meat products

2013



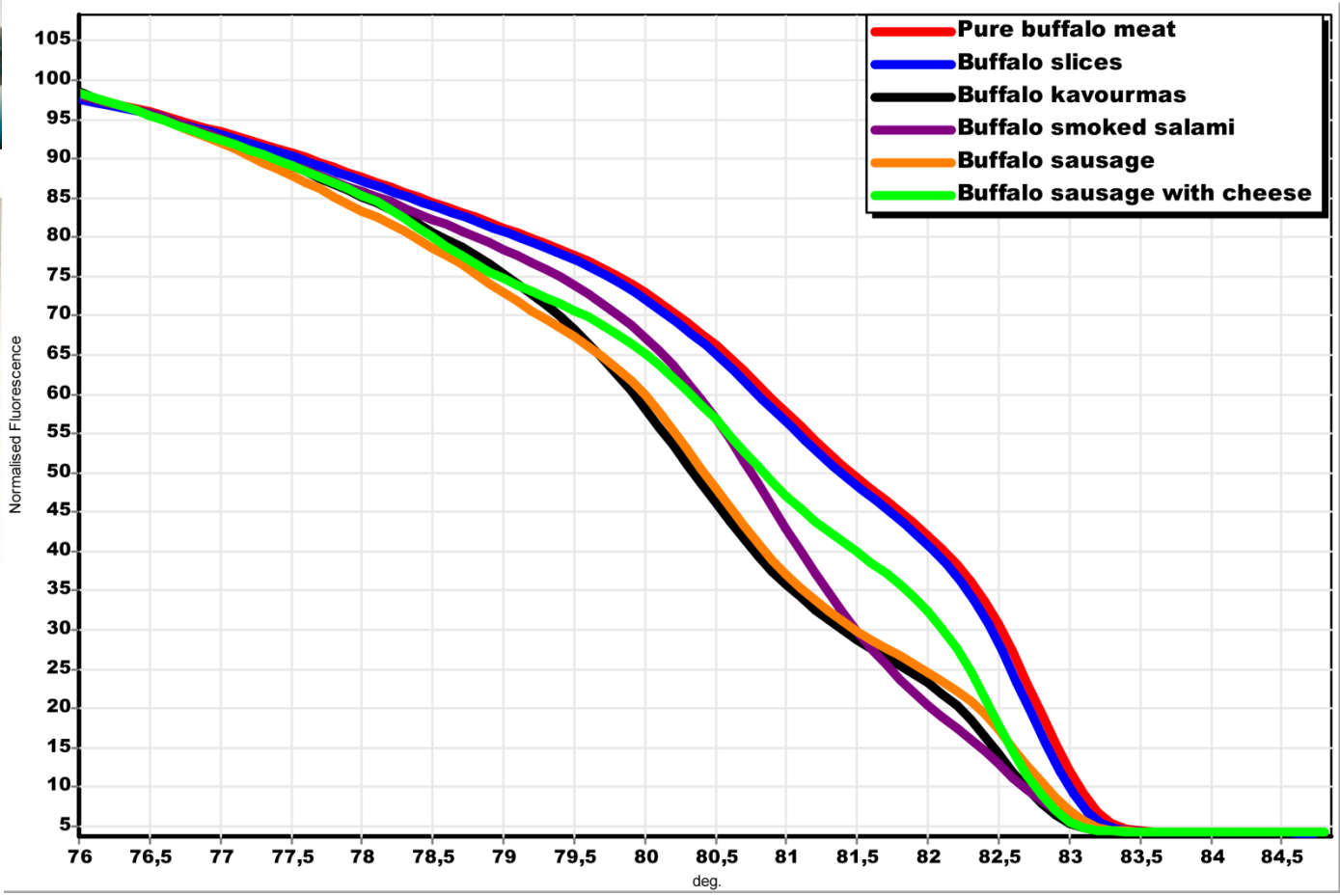
Buffalo meat products

2013

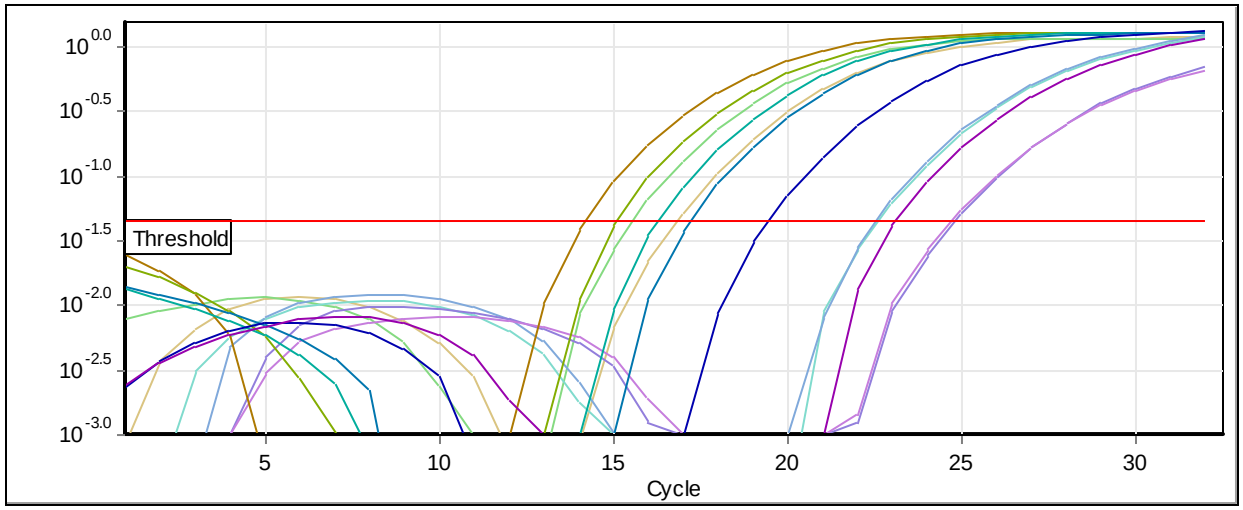


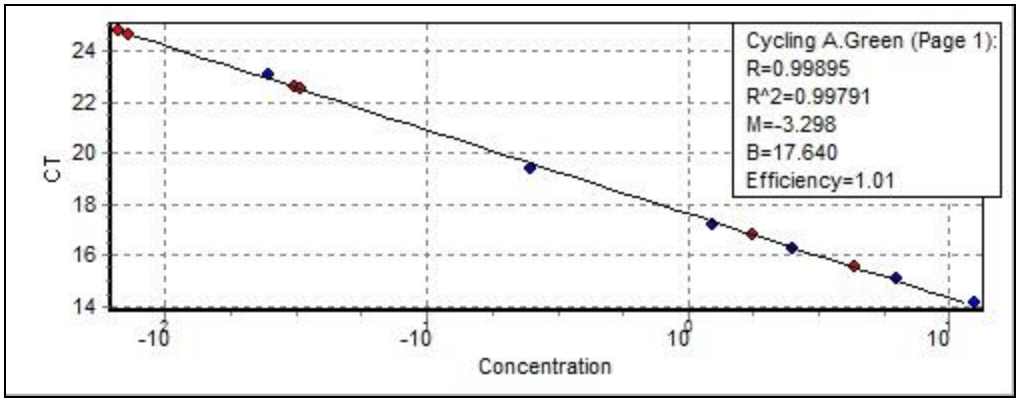
Buffalo meat products

2013



Detection of Horse DNA in Meat products

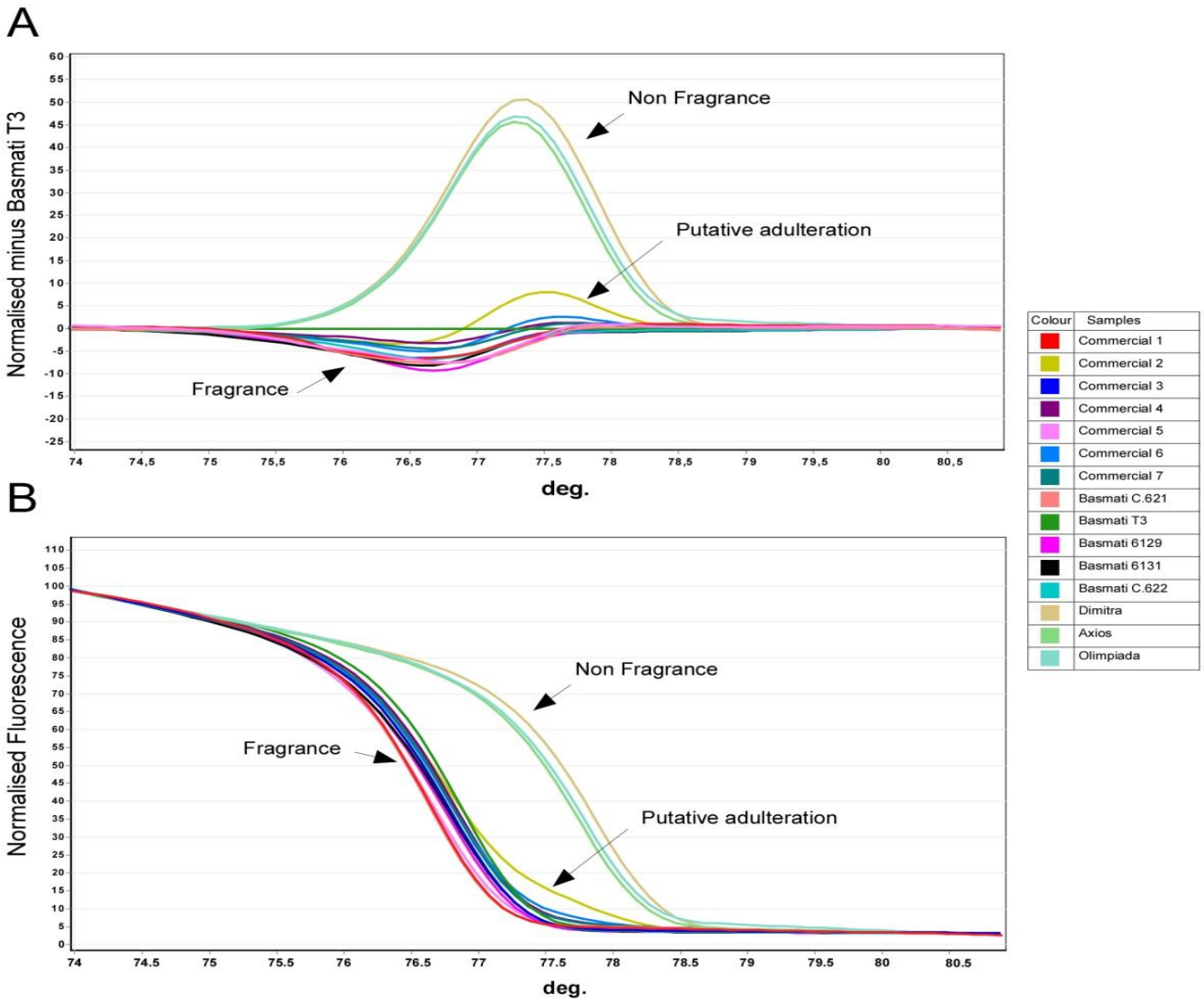




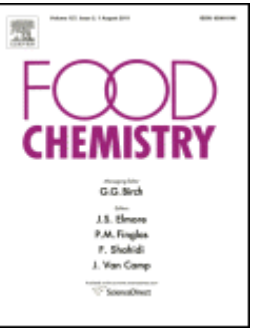
Available also for:

- Donkey
- Mule
- Chicken
- Turkey
- Pig (Halal and Kosher certification)

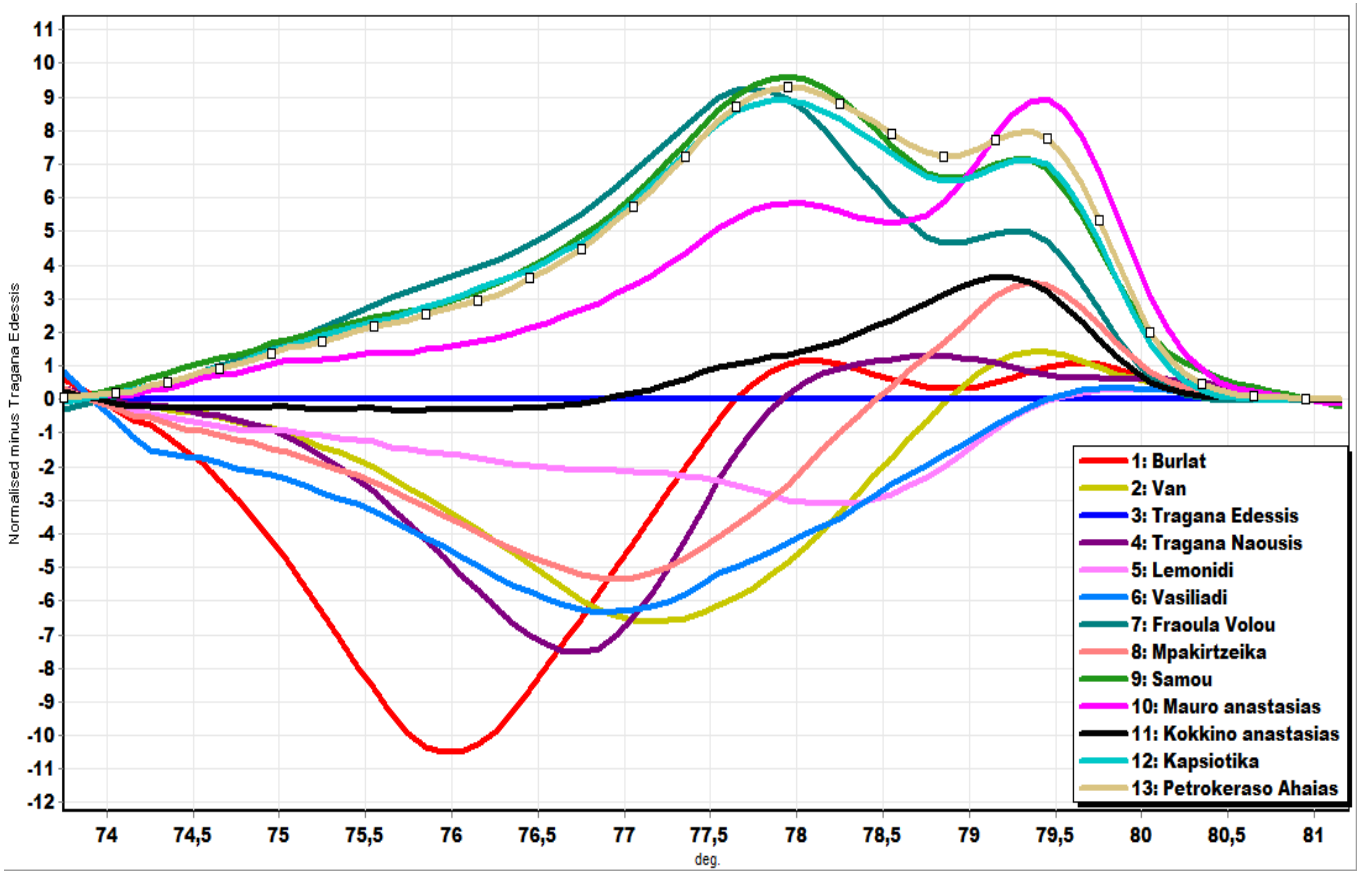
Basmati rice



2011

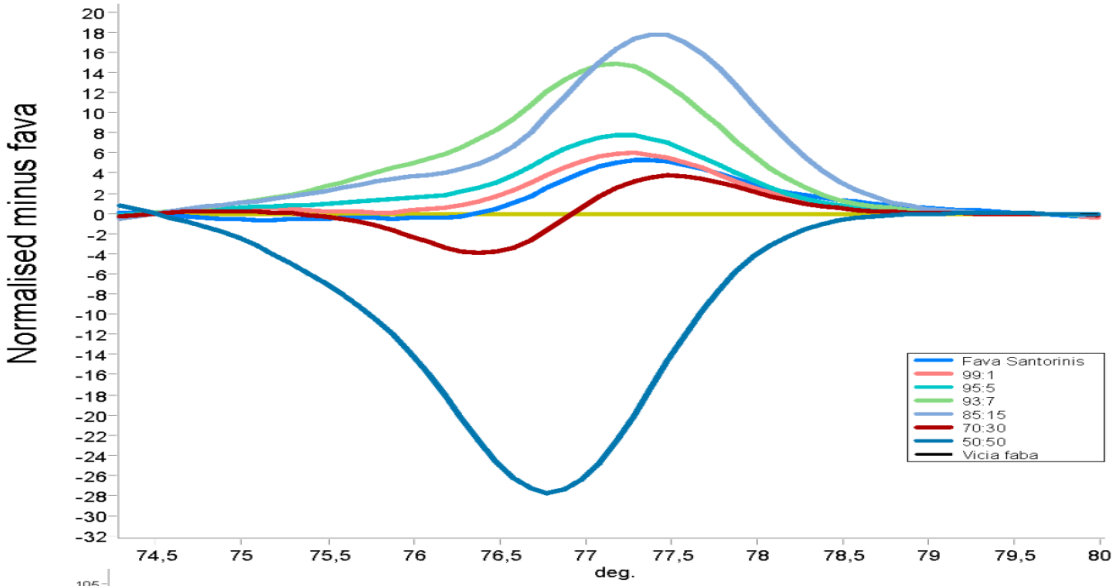


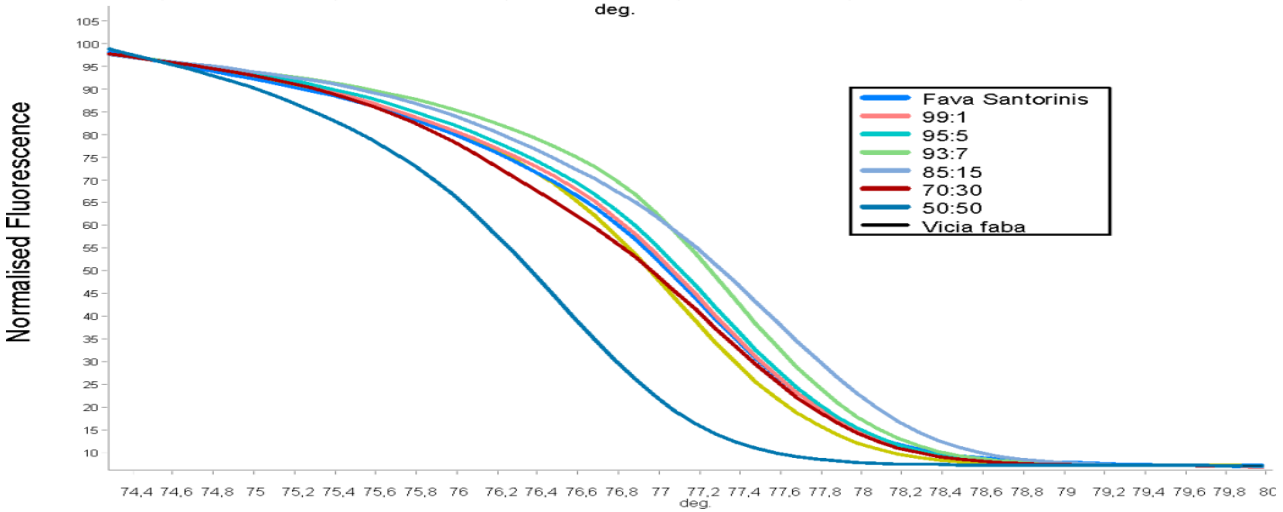
Sweet Cherries



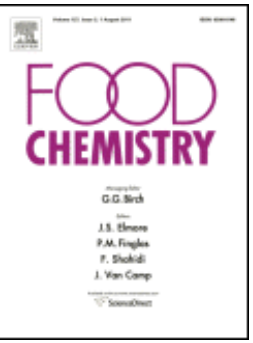
2011

Fava of Santorini

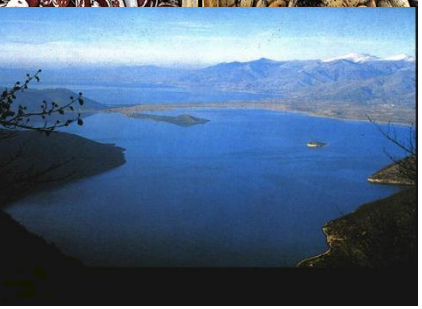




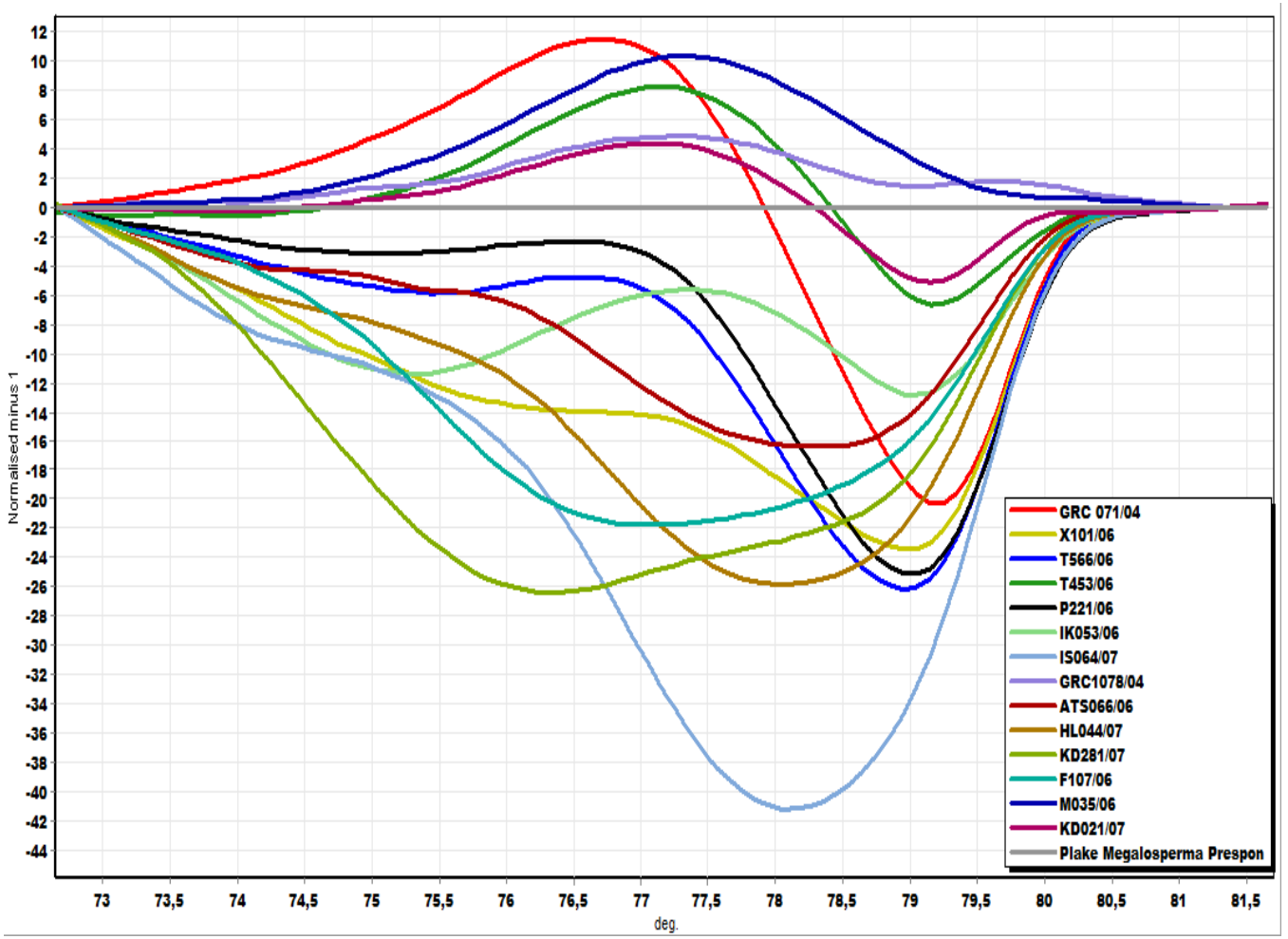
2012



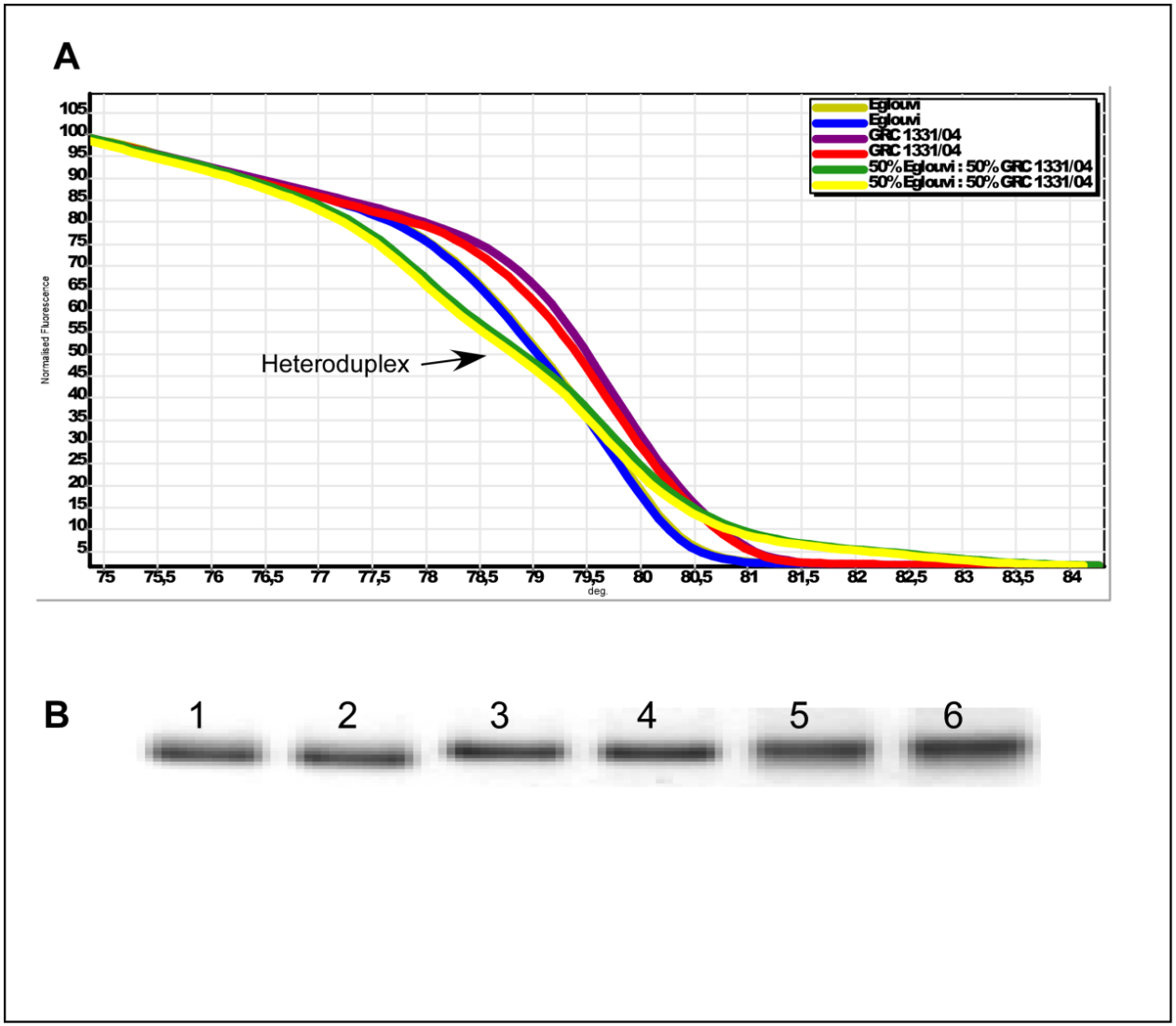
Beans of lake Prespa



2012

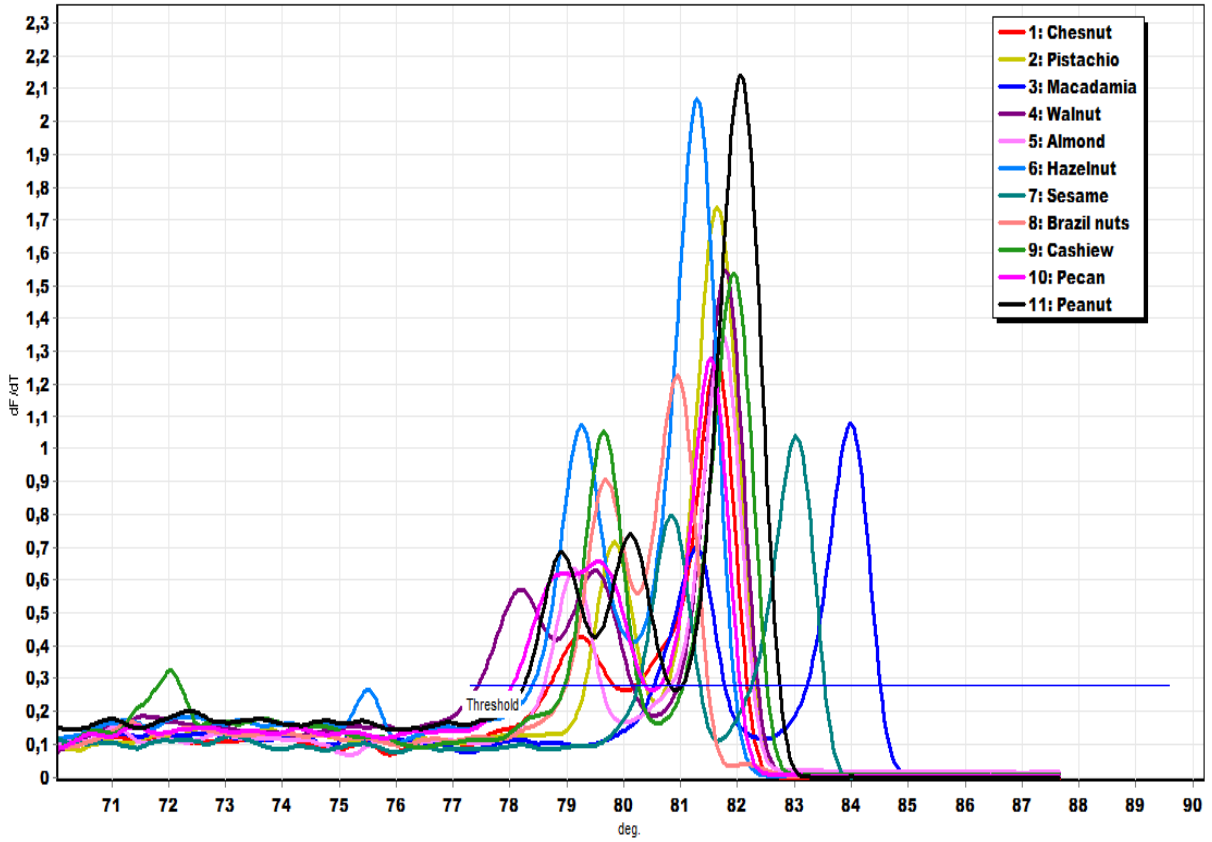


Lentils of Englouvi



2012

Nuts


1: Chesnut
2: Pistachio
3: Macadamia
4: Walnut
5: Almond
6: Hazelnut
7: Sesame
8: Brazil nuts
9: Cashiew
10: Pecan
11: Peanut
Threshold
dF /dT
deg.

2012


CONTROL
CONTROL
FOOD CONTROL
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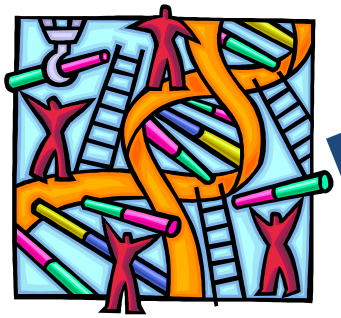
CONTROL
CONTROL
CONTROL

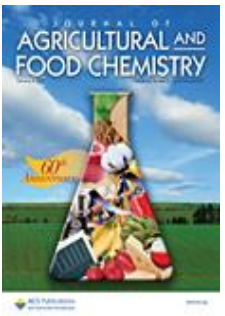
Olive Oil

Isolation of DNA

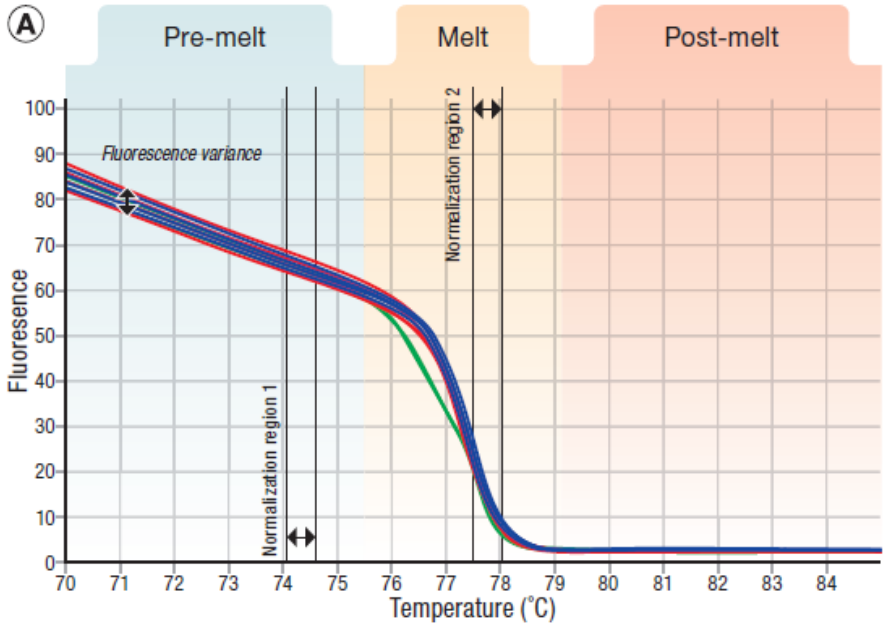


Analysis

2012



Genotyping based on chloroplastic markers



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FOOD CONTROL
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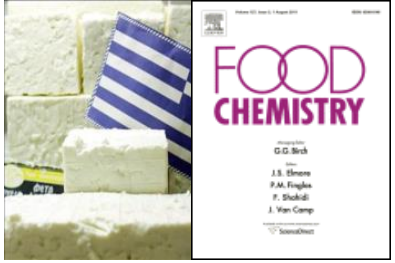
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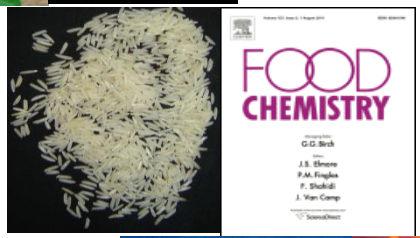
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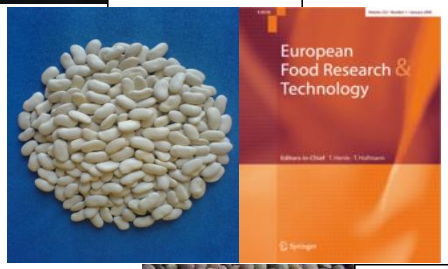
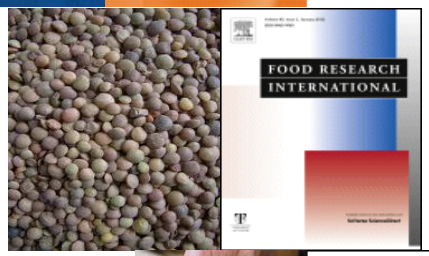

European
Food Research &
Technology

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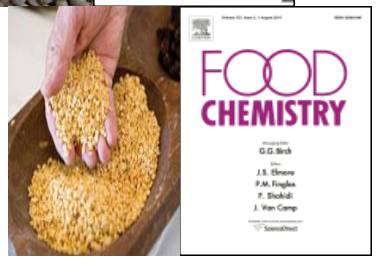
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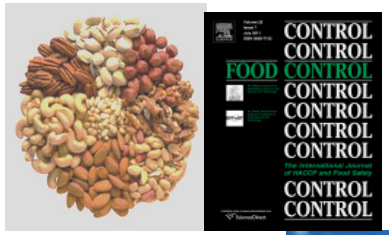

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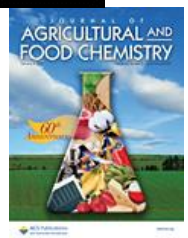
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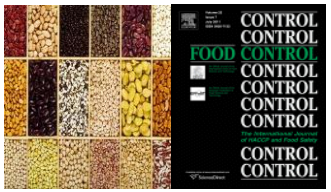
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WORK IN PROGRESS

Conclusions

- Many DNA methods available for Food Traceability and detection of adulterations
- Most of them require high cost equipment, high expertise and are time consuming
- Most promising (in our lab):
  - HRM (requires RT-PCR system with HRM capabilities)
- **Future trends:**
  - Low cost Next Generation Sequencing (NGS) (based on biosensors?)
  - Biosensors

Identification of bioprotective Lactic acid bacteria using High Resolution Melting (HRM) analysis

Protective cultures

Antagonistic microorganisms added to food products:

- to inhibit pathogens
- to prolong shelf life
- while affecting the sensoric properties as little as possible

Lactic acid bacteria (LAB) are ideal choice for application as protective cultures since:

- they are frequently naturally present in food products
- form part of the gut microflora of humans and animals
- have a long history of safe use

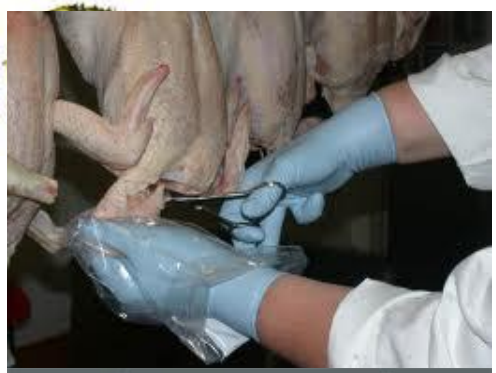
Materials and Methods

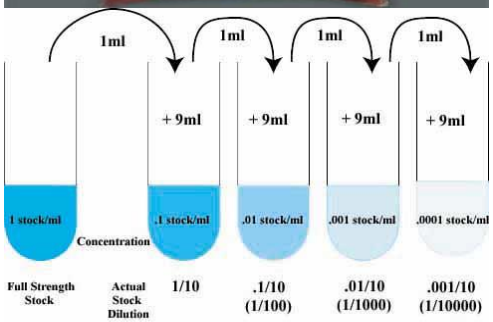
Sampling

- 4 poultry slaughterhouses of N. Greece
- Sampling after the slaughter process
- 10g of neck skin
- Each sample: 25g of neck skin, from 3 broiler carcasses (EN/ISO 17604:2003)

Lactic acid bacteria

- Total samples = 100 (300 carcasses)
- Double Layer Inhibition method
- Selection of psychrothophic LAB
- Detection of biogenic amines production (HPLC)
- Biochemical identification (API 50 CH)
- Molecular identification (Sequencing of 16S-23S ISR region, HRM analysis)





Isolation

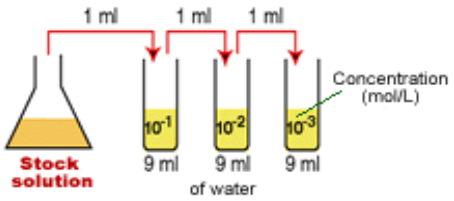
Sample (25 g)

Homogenization: 225ml Peptone dilution water (2 min)
Revivification: Room temperature for 30 min

4 decimal dilutions
Inoculation of MRS agar
Incubation at 7˚C for 10-15 days

Selection of petri dishes MRS agar showing 10-30 colonies.

Double Layer Inhibition method

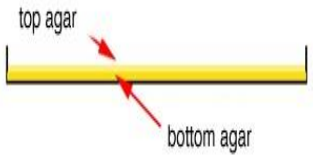


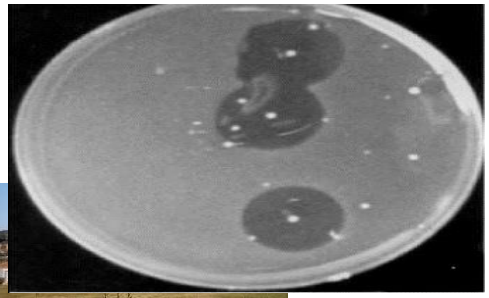
Salmonella spp. in BHI
37˚C for 48 h

Listeria monocytogenes in BHI
37˚C for 48 h

3 decimal dilutions
Inoculation in 15 ml of molten soft BHI agar

Topping of MRS agar showing 10-30 colonies of presumptive LAB with the semi soft BHI agar containing *Salmonella* and *Listeria*
Incubation at 37˚C for 48 ώρες

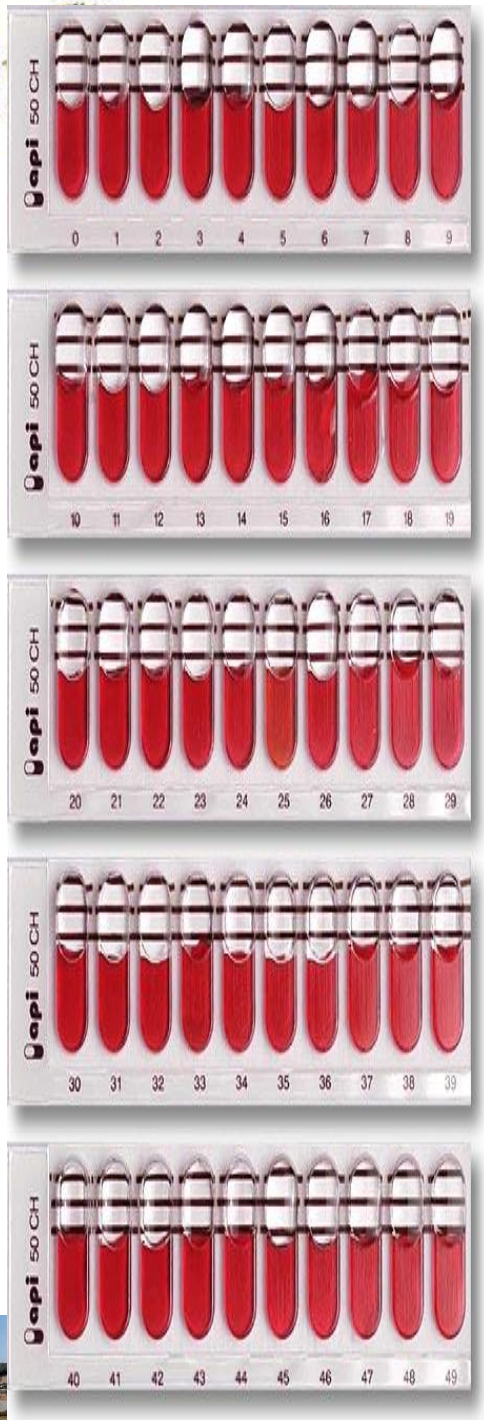




Selection of presumptive LAB colonies from MRS agar that presented inhibition zone in BHI agar

Selection of psychrothophic LAB

- Gram(+), catalase (-), oxidase(-)
- Incubation at 7˚C for 10 – 15 days



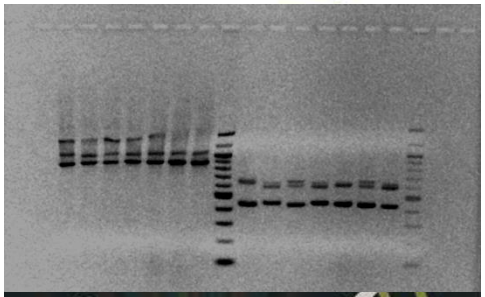
Biochemical identification

API 50 CH biochemical test strip

(Fermentation of 49 carbohydrates)

The 50 most efficient isolates of presumptive LAB were selected based on:

- Diameter of inhibition zone
- Ability to grow at 7˚C
- Concentration of LAB and pathogens (lowest conc. of LAB vs highest conc. of pathogens)



Molecular identification

- One isolate from each species (biochemical identification)
- Selection: highest percentage of API certainty
- Extraction of DNA
- PCR (16s-23s rRNA, Intergenic Spacer Region, ISR)
- Electrophoresis
- Sequencing of DNA and analysis with BLAST algorithm

High Resolution Melting (HRM) analysis

- 50 isolates of LAB identified with API 50 CH
- Revivification in MRS broth and inoculation on MRS agar
- Extraction of DNA (tissue kit Macherey Nagel)
- PCR (16s rRNA)
- HRM analysis vs API 50 CH vs sequencing

Results

Isolation of psychrotrophic LAB

- 92 isolates of LAB were obtained (Gram positive, catalase/oxidase negative)
- 83 isolates of LAB presented growth at 7ºC

Biochemical identification of LAB

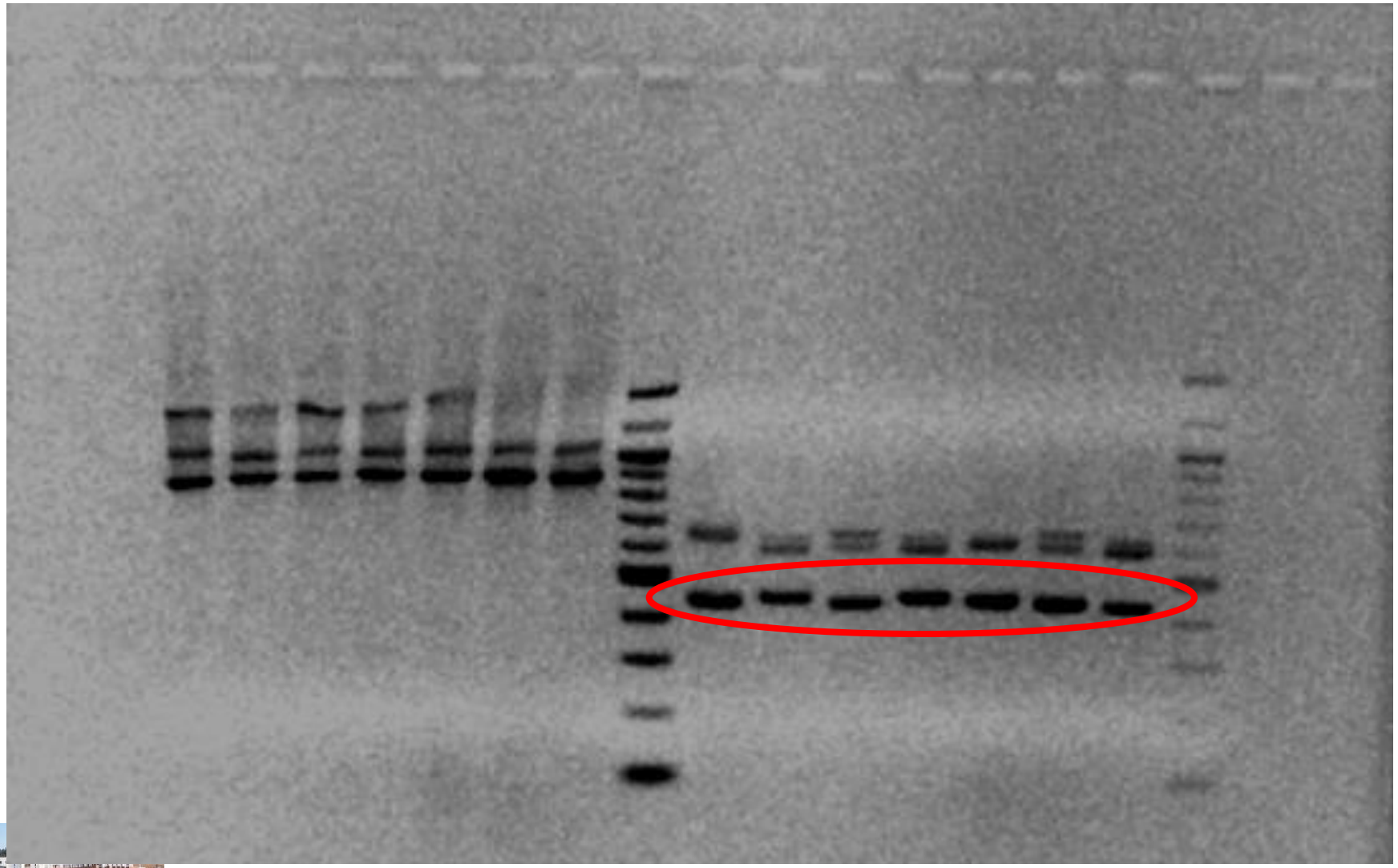
- 7 species of LAB *Leuconostoc lactis, Lactobacillus salivarius, Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus brevis, Pediococcus acidilactici*)

Biochemical identification of LAB

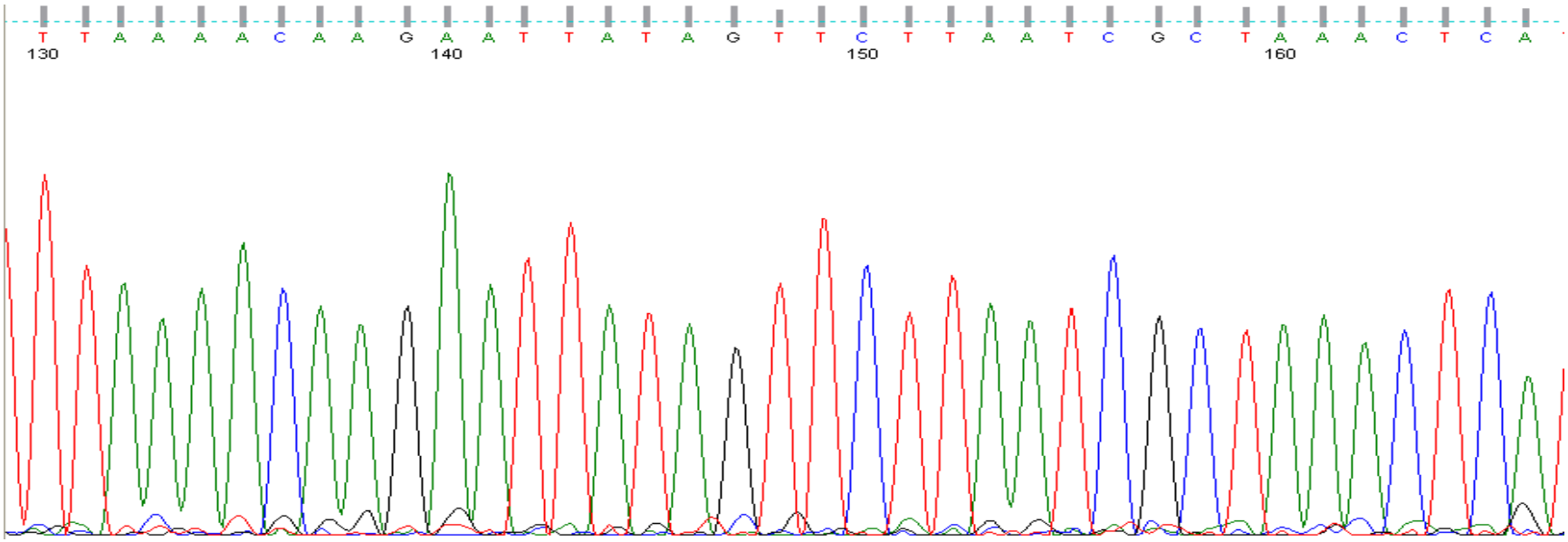
Identification with API 50 CH	Number of isolates	Certainty %
Leuconostoc lactis	19	60,2-96,9%
Lactobacillus salivarius	9	98,7-99,9%
Lactobacillus fermentum	8	30,9-99%
Lactobacillus delbrueckii	7	55,7-97,6%
Lactobacillus acidophilus	3	68,5-98,4%
Lactobacillus brevis	3	59,1-98,3%
Pediococcus acidilactici	1	99,9%

Molecular identification of LAB

PCR of ISR region and electrophoresis



Sequencing of ISR region



LAB_59

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Identification with BLAST algorithm

>gb|CP000233.1| *Lactobacillus salivarius* UCC118, complete genome
Length=1827111
rRNA-23S ribosomal RNA

Identities = 741/743 (99%), Gaps = 0/743 (0%)

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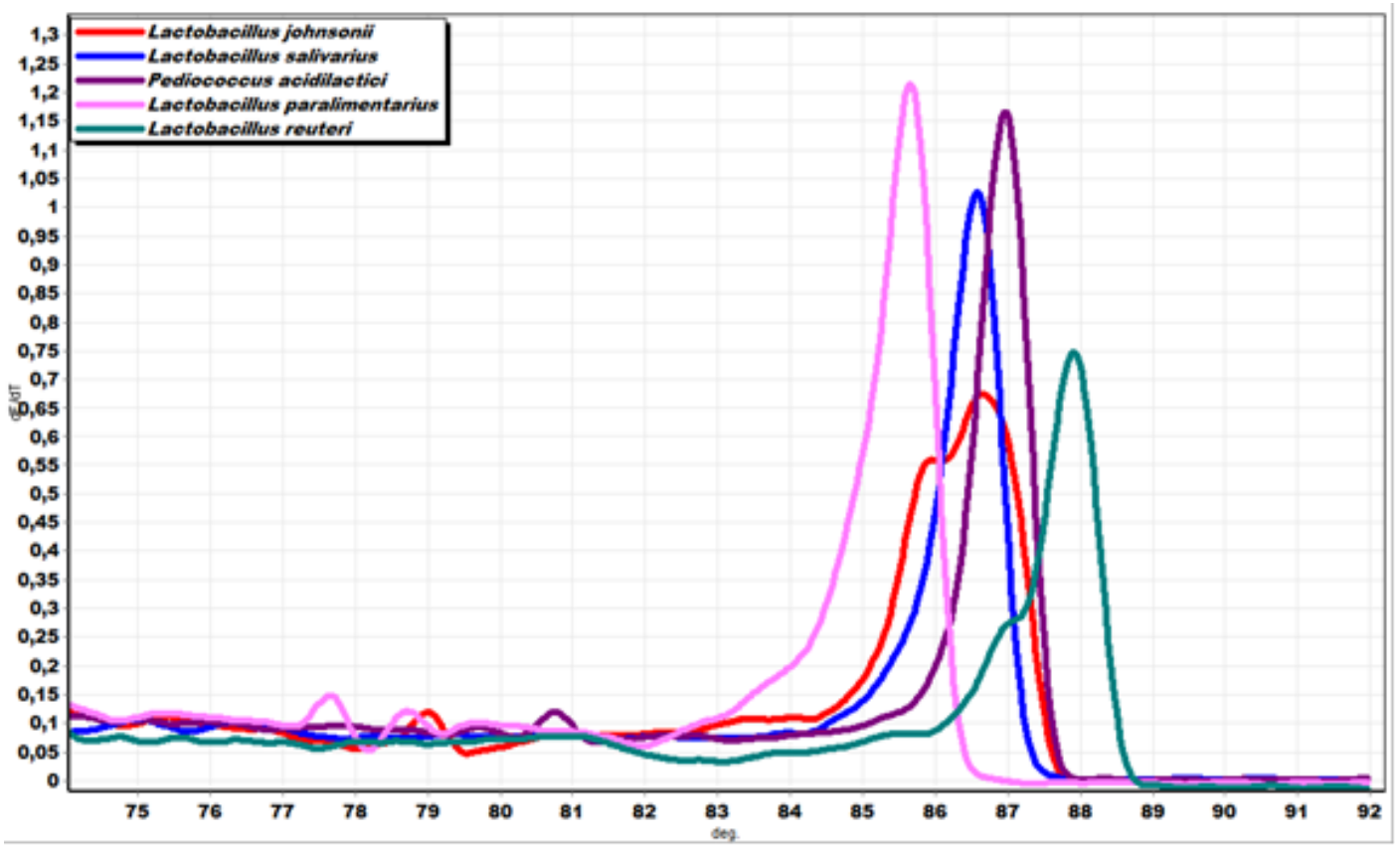
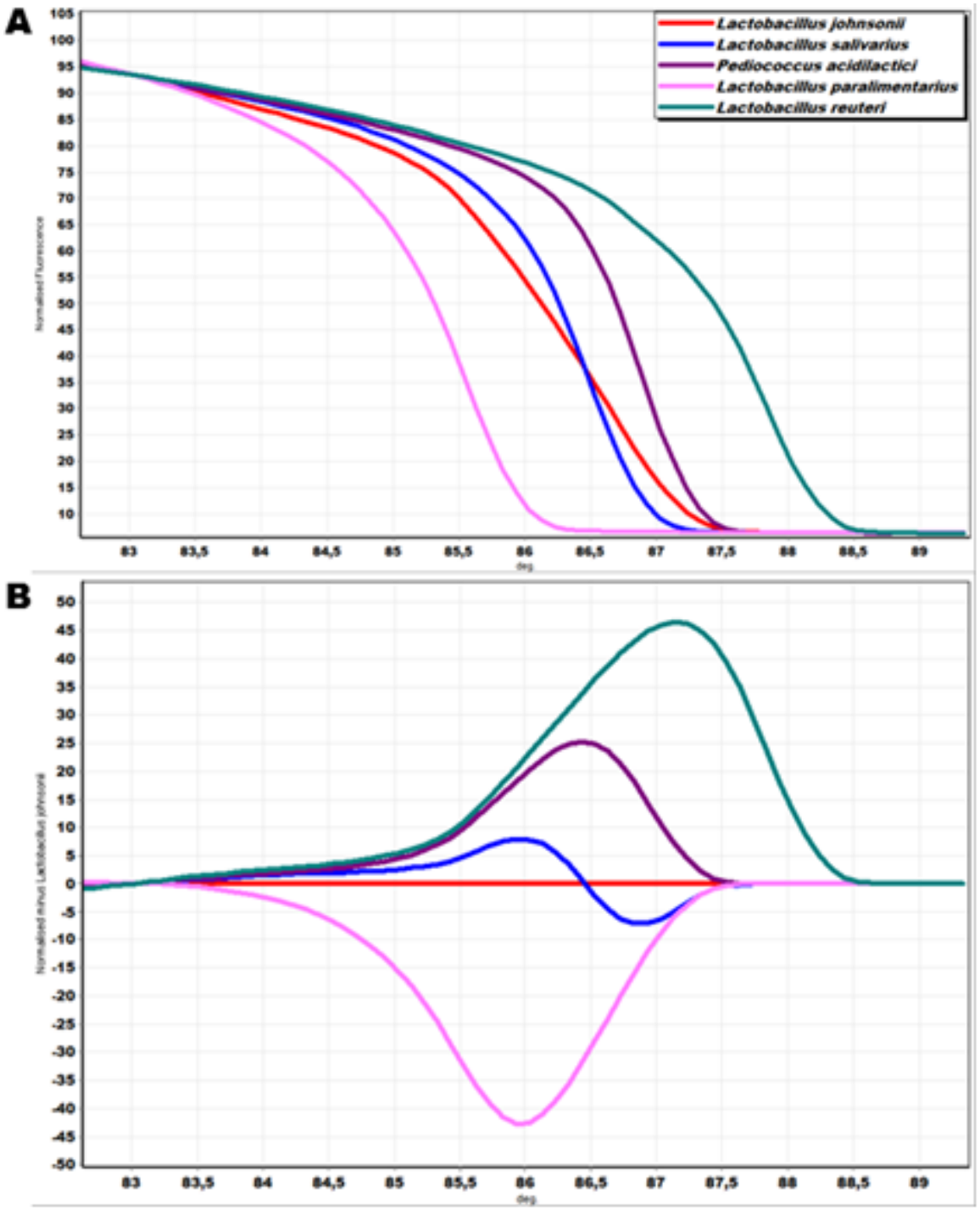
Comparative results of molecular and biochemical identification

	Identification with DNA sequencing		Identification with API 50 CH	
Isolates	**Species**	**% Similarity**	**Species**	**% Certainty**
5	*Lactobacillus johnsonii* NCC 533	99,64%	*Lactobacillus acidophilus*	96,6%
7	*Pediococcus acidilactici* DSM 20284	99,79%	*Lactobacillus delbrueckii*	97,6%
40	*Lactobacillus salivarius* CECT 5713	99,64%	*Leuconostoc lactis*	96,9%
48	*Pediococcus acidilactici* clone P10	100%	*Pediococcus acidilactici*	99,9%
51	*Lactobacillus paralimentarius* DSM 13238	99%	*Lactobacillus brevis*	98,3%
59	*Lactobacillus salivarius* CECT 5713	99,86%	*Lactobacillus salivarius*	99,9%
74	*Lactobacillus reuteri* DSM 20016	100%	*Lactobacillus fermentum*	98%

Species	Peak 1	Peak 2
Lactobacillus johnsonii	85.95±0.1	86.65±0.3
Lactobacillus salivarius	86.58±0.1	
Pediococcus acidilactici	86.97±0.2	
Lactobacillus paralimentarius	85.65±0.2	
Lactobacillus reuteri	87.9±0.1	




A
Lactobacillus johnsonii
Lactobacillus salivarius
Pediococcus acidilactici
Lactobacillus paralimentarius
Lactobacillus reuteri
Normalised Fluorescence
105
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deg

	Isolates	IDENTIFICATION		
		API 50CH	SEQUENCING	HRM
1	**5**	***Lactobacillus acidophilus***	***Lactobacillus johnsonii***	***Lactobacillus johnsonii***
2	**7**	***Lactobacillus delbrueckii***	***Pediococcus acidilactici***	***Pediococcus acidilactici***
3	27	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
4	**40**	***Leuconostoc lactis***	***Lactobacillus salivarius***	***Lactobacillus salivarius***
5	41	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
6	42	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
7	43	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
8	45	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
9	46	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
10	47	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
11	**48**	***Pediococcus acidilactici***	***Pediococcus acidilactici***	***Pediococcus acidilactici***
12	49	*Lactobacillus brevis*	-	*Lactobacillus salivarius*
13	**51**	***Lactobacillus brevis***	***Lactobacillus paralimentarius***	***Lactobacillus paralimentarius***
14	53	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
15	54	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
16	55	*Leuconostoc lactis*	*Lactobacillus salivarius*	*Lactobacillus salivarius*
17	56	*Lactobacillus salivarius*	*Lactobacillus salivarius*	*Lactobacillus salivarius*
18	58	*Lactobacillus salivarius*	-	*Lactobacillus salivarius*
19	**59**	***Lactobacillus salivarius***	***Lactobacillus salivarius***	***Lactobacillus salivarius***
20	63	*Lactobacillus salivarius*	*Lactobacillus salivarius*	*Lactobacillus salivarius*
21	64	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
22	65	*Lactobacillus salivarius*	-	*Lactobacillus salivarius*
23	66	*Lactobacillus salivarius*	-	*Lactobacillus salivarius*
24	68	*Lactobacillus salivarius*	-	*Lactobacillus salivarius*
25	69	*Leuconostoc lactis*	-	*Lactobacillus salivarius*
26	70	*Lactobacillus salivarius*	-	*Lactobacillus salivarius*
27	71	*Lactobacillus fermentum*	-	*Lactobacillus reuteri*
28	73	*Lactobacillus fermentum*	-	*Lactobacillus reuteri*
29	**74**	***Lactobacillus fermentum***	***Lactobacillus reuteri***	***Lactobacillus reuteri***

Conclusions

- Several isolates of psychrotrophic LAB were obtained from chicken carcasses with potential inhibitory activity against *Salmonella* and *Listeria*

- These LAB are part of the natural microflora of carcasses

- The biochemical identification was proven relatively unreliable, but still useful

- HRM analysis was found reliable and its results were identical to those from DNA sequencing

HRM analysis:

- Was found suitable for identification of a large number of LAB isolates
- Low cost
- Genotyping of the isolates according to their melting profile
- Sequencing of only one isolate from each group of LAB with a common melting profile
- The melting profile of identified LAB can be used as a reference for the identification of unknown LAB species without the need for DNA sequencing

Thank you for your attention