



Cow's milk allergens: Screening gene markers for the detection of milk ingredients in complex meat products

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ABSTRACT

Cow's milk proteins are food allergens present in uncounted foodstuffs. By exploiting and comparing different regions of bovine genome, this work intended to develop a real-time PCR method to detect/quantify traces of milk in complex foods. Two mitochondrial (*cytb* and 12S rRNA) and two nuclear (β -lactoglobulin and β -casein) genes showed the most promising results by qualitative PCR. However, real-time PCR assays targeting nuclear genes revealed cross-reactivity with some meat species, while the 12S rRNA assay was the most specific (only minor reactivity with two species above 36 cycles of amplification), providing a linear dynamic range of 10–0.05% of cow's milk protein concentrate (MPC) in raw meat and cooked ham, with acceptable performance parameters. The results highlight the potentiality of the assay for the development of a novel method to quantify milk ingredients in meat products.

1. Introduction

Milk is one of the eight groups of foods accountable for about 90% of food-allergic reactions. Cow's milk allergy is a common food allergy in early childhood, with an incidence estimated from 2.0% to 7.5% in the first year of life (Mousan & Kamat, 2016). It is typically mediated by immunoglobulin E (IgE), inducing adverse immunological reactions to milk proteins in sensitised/allergic individuals, even when ingested at trace amounts (Monaci, Tregoat, van Hengel, & Anklam, 2006; Villa, Costa, Oliveira, & Mafra, 2018). β -Lactoglobulin, caseins and α -lactalbumin are the most abundant proteins in cow's milk, being also classified as major allergens (Monaci et al., 2006; Villa, Costa, Oliveira, & Mafra, 2018).

Besides the well-known dairy products, such as milk beverages, cheeses and yogurts, milk proteins are present in a great number of processed foods, namely meat and fish products, desserts and bakery products, among others. Enriched milk powders, such as milk protein concentrates (MPC, protein content < 90%) or milk protein isolates (MPI, protein content > 90%), are typical dairy ingredients used by the food industry due to their functional properties (Fox, 2001; Meena, Singh, Panjagari, & Arora, 2017). MPC are described as concentrated forms of milk proteins that contain both caseins and whey proteins in the same proportions as the whole milk, providing an increased heat stability, solubility and gel-forming capacity to food products (Uluko, Liu, Lv, & Zhang, 2016).

The accidental exposure to these proteins is recurrent, posing allergic individuals to a constant threat. Therefore, the EU has established specific legislation requiring the mandatory labelling of fourteen groups of foods (thirteen potentially allergenic), including milk and dairy products, that must be emphasised over the rest of the ingredients enumerated in processed food labels, regardless of their quantity (Directive 2007/68/EC; Regulation (EU) No 1169/2011). However, mislabelling or unintentional cross-contaminations during food processing can occur, making the development of analytical methodologies with high specificity and sensitivity a demanding task.

Presently, there several methods for the detection of milk allergens in processed foodstuffs, targeting allergenic proteins by enzyme-linked immunosorbent assay (ELISA) (de Luis, Lavilla, Sánchez, Calvo, & Pérez, 2009; Decastelli, Gallina, Manila Bianchi, Fragassi, & Restani, 2012; Deckwart et al., 2014) and mass spectrometry (MS) platforms (Boo, Parker, & Jackson, 2018; Jira & Schwägele, 2015; Pilolli, De Angelis, & Monaci, 2017; Pilolli, De Angelis, & Monaci, 2018; Planque et al., 2019). More recently, DNA-based methods have revealed as promising techniques in allergen detection due to their high specificity and sensitivity, taking advantage of their applicability to processed food analysis because of the high thermal stability of DNA molecules (Costa, Fernandes, Villa, Oliveira, & Mafra, 2017a). Some DNA-based methods have been developed targeting milk allergen-encoding genes, such as α -lactalbumin (Xiao, Qin, Wenju, & Qin, 2016) or mitochondrial genes (Köppel et al., 2010; Köppel, Velsen-Zimmerli, & Bucher,

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Table 1

Key data of primers designed to specifically target DNA sequences from mitochondrial and nuclear genome of cow.

Target region	Primers	Sequence (5' → 3')	Amplicon (pb)	Concentration (nM)	Annealing temperature (°C)	NCBI (Accession no.)	Reference
cyt b	Bos-F	CTGCCGAGACGTGAACACG	99	160	65	D34635.1	Amaral, Santos, Melo, Oliveira, and Mafra (2014)
	Bos-R	AAGCCTCGTCCTACGTGCATA					
	Bcyt-F	ACGGGTCTTACACTTTCTAGAAAC	139	240	65	AY526085.1	This work
	Bcyt-R	AAGAGGTGGTGATGACTGTTGCT	115	240	60	KF926377.1	This work
	Beef-F	CCTTCTCTATCCTAATTCTTGCTC					
	Beef-R	AGTAGGTCTGCTACTAGGGC	124	240	60	KF926377.1	This work
	B-F1	CCTAGCCTTCTCTATCCTAATTC					
	B-R1	GTGAGTGTCACTAGGTCTGCTA	112	240	60	KF926377.1	This work
	B-F2	TTCTTGCTCTAATCCCCCTACT					
	B-R2	CATGTGAGTGTCACTAGGTCTG	256	200	55	AY526085.1	Bottero et al. (2003)
12S rRNA	916	GTACTACTAGCAACAGCTTA					
	1171	GCTTGATTCTCTTGGTGTAGAG	121	200	56	AY526085.1	This work
	916	GTACTACTAGCAACAGCTTA					
	916-R	AGACTGTATTAGCAAGAATTGGTG	123	160	56	AY526085.1	This work
	916-F	AGAGTACTACTAGCAACAGCTTA					
	916-R	AGACTGTATTAGCAAGAATTGGTG	117	160	56	AY526085.1	This work
	916-F1	CTACTAGCAACAGCTTAAACTC					
	916-R	AGACTGTATTAGCAAGAATTGGTG	170	240	60	AY526085.1	This work
	B12S-F	CTACACCAAGAGAATCAAGCACG					
	B12S-R	GTGCGTTTAAATAGGGTTAGATGC	163	240	60	AY526085.1	This work
COI	mit-F	TCTACACCAAGAGAATCAAGCA					
	mit-R	CCAAGAGAATCAAGCACGAAAGT	157	240	60	AY526085.1	This work
	mit-F2	CCAAGAGAATCAAGCACGAAAGT					
	mit-R	CCAAGAGAATCAAGCACGAAAGT	140	160	65	AY526085.1	This work
	BCOI-F	TACTCTCGTCCCTGTATTAGCA					
	BCOI-R	TTCGGGGTGTCCAAAGAATCAGA	146	240	65	AY526085.1	This work
	Bmt-F1	AGCCATATATCTCCTTGGCTGACA					
	Bmt-R	TGGTGTCACTTCTGGATTGTGATA	140	240	65	AY526085.1	This work
	Bmt-F2	TATACTCTCCTTGGTGACATGCC					
	Bmt-R	TGGTGTCACTTCTGGATTGTGATA	147	160	65	AY526085.1	This work
tRNA-Lys/ATP synthase F0 subunit 8	tRD-F	AACTGCAGTCTCACCATCAACC					
	tRD-R	GGGTGCAAAATCTGTGTTGAGTTA	149	240	60	AY526085.1	This work
NADH subunit 2	ND2-F	ACTCACGTATTCTACCACTAAC					
	ND2-R	CTGATAGTATTGGCGTGAGTGGT	105	240	62	AY526085.1	This work
NADH subunit 4	ND4-F	GTAAATATAGTAATCACC GCCCTA					
	ND4-R	CCGTGTAAGGAAGGCGAGATA	118	240	62	AY526085.1	This work
NADH subunit 4L	ND4L-F	CACAGTATCTCTTGTAGGACTAC					
	ND4L-R	TGAGTTGAGGATTGTTAGGGCTG	106	200	64	XM_005902037.2	This work
β-casein	BCAS-F1	CACCAGCCTCTTCTCCAACTG					
	BCAS-R2	TCTGGGGATAGGGCACTGCTTT	120	200	65	Sequencing data	This work
β-lactoglobulin	BLG-F1	GCTGGCCACATGCTCGCTCT					
	BLG-R1	GTGTCCACTTCTGAGCCTC					

2012), as molecular markers for milk detection in foods. However, DNA-based methods focusing on the detection of cow's milk ingredients in meat products, such as milk protein concentrates, which are frequently used in the preparation of hams and sausages (Spychaj, Pospiech, Iwańska, & Montowska, 2018), are still lacking.

In this context, the present work intended to investigate different mitochondrial and nuclear regions as potential DNA markers for the development of a highly specific and sensitive PCR method. Different regions of bovine genome (mitochondrial vs allergen-encoding genes) were extensively exploited and compared in order to develop a method able to detect trace quantities of cow's milk in complex/processed foods. The selected regions were used in the development of qualitative PCR and real-time PCR assays, evaluating their sensitivity and specificity with the final goal of selecting the best molecular marker for the detection/quantification of cow's milk as a potential allergenic food in processed meat products.

2. Materials and methods

2.1. Sampling

Two sets of binary model mixtures containing 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% (w/w) of MPC in turkey meat were prepared. To simulate ham

preparation, turkey meat was previously minced using a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany), with the addition of 8 g of salt and 4 g of powder sugar in a total of 1 kg. To facilitate homogenisation, a sterile phosphate-buffered saline solution (0.2 M) was added to this mixture. MPC was provided by FORMULAB (Maia, Portugal) and the turkey meat (muscle) was acquired at a local retail market. The exact milk protein content of MPC was determined by the Kjeldahl method, corresponding to 83.4%. Taking into consideration this value, the first mixtures containing 10% of MPC were prepared by adding the required amount (24.0 g) to minced turkey meat (mixture for ham) (in a total of 200 g). The other model mixtures were prepared by successive additions of the mixture for ham. A set of binary mixtures was cooked in the oven at 67 °C for 5 h simulating ham production, while the other set remained raw.

For assay specificity testing, 15 different meat species were tested: boar, duck, partridge, hare, quail, pheasant, deer, rabbit, chicken, turkey, lamb, goat, ostrich, horse and pork.

To avoid contaminations, all mixtures and meat samples were homogenised separately, and all materials and different containers were previously treated with a DNA decontamination solution. All mixtures and meat samples were immediately stored at −20 °C until further analysis.

2.2. DNA extraction

The NucleoSpin food kit (Macherey-Nagel, Düren, Germany), with some minor modifications, was the selected protocol to extract the DNA from the binary model mixtures and from MPC. DNA from meat species was extracted using the Wizard method as described by Mafra, Silva, Moreira, da Silva, and Oliveira (2008). The extractions were performed at least in duplicate assays using 200 mg of each sample. The extracts were kept at -20°C until further analysis.

To evaluate the yield and purity of DNA extracts, UV spectrophotometric DNA quantification was performed on a SynergyHT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take 3 micro-volume plate accessory. The DNA content was assessed by the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA).

2.3. Oligonucleotide primers and probes

The primers used in this work were designed on different sequences of mitochondrial and nuclear genes of *Bos taurus* retrieved from NCBI database or previously reported in the literature (Table 1). Both hydrolysis probes, targeting the mitochondrial 12S rRNA and *cytb* genes sequences of cow (916-P – FAM-TCTAGAAGGATATAAAGCACCGCCA AGT-BHQ1 and Bos-P – FAM-CCGATACATACAGCAAACGGAGCTT CAA-BHQ1, respectively), were specifically designed for this work and labelled with FAM as fluorescent reporter and BHQ-1 as quencher. All the primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

For specificity purposes, *in silico* analysis of the nucleotide and primer sequences was performed using the basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), respectively. OligoCalc software (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) was also used to check primer properties and to ensure the absence of primer hairpins and self-hybridisation.

To evaluate the amplification capacity of extracts, all the samples were amplified by qualitative PCR using universal eukaryotic primers (18SRG-F – CTGCCCTATCAACTTTCGATGTA and 18SRG-R – TTGGA TGTGGTAGCCGTTTCTCA) targeting a sequence of 113 bp of a highly conserved 18S rRNA nuclear region (NCBI accession no. [HQ873432.1](#)) (Costa, Oliveira, & Mafra, 2013).

2.4. Qualitative PCR

The PCR amplifications were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total reaction volume of 25 μL , containing 2 μL of template DNA (40 ng for binary mixtures and 100 ng for meat samples), 1 $\times$ buffer (67 mM of Tris-HCl (pH 8.8), 16 mM of $(\text{NH}_4)_2\text{SO}_4$, 0.1% of Tween 20), 200 μM of each dNTP (Grisp, Porto, Portugal), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl_2 (2.0 mM for primers BLG-F1/BLG-R1 and 1.5 mM for primers 18SRG-F/18SRG-R), and 160–240 nM of each primer (according to Table 1). The amplification programmes were defined as following: initial denaturation at 95°C for 5 min; 40 cycles (except for primers 18SRG-F/18SRG-R with 33 cycles and 43 cycles for both sets of primers BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2) at 95°C for 30 s, 55 – 65°C (according to Table 1) for 30 s (except for primers 916–1171 for 60 s) and 72°C for 30 s; and a final extension at 72°C for 5 min.

PCR products were verified by electrophoresis in a 1.5% agarose gel stained with GelRed 1 $\times$ (Biotium, Inc., Hayward, CA, USA) and carried out in 1 $\times$ SGTB (Grisp, Porto, Portugal) for 25–30 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using Image Lab software version 5.2.1 (Bio-Rad Laboratories,

Hercules, CA, USA). Each extract was amplified at least in two independent assays.

2.5. Sequencing

β -Lactoglobulin region of cow (NCBI accession no. X14712.1) was selected to be sequenced. This region was amplified with primers BLG-F – GCCACATCTAGGTGAGCCCT and BLG-R – AGGGGTGAATGTGGTC CCGGTT producing fragments of 520 bp with the following amplification conditions: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 45 s, 65°C for 45 s and 72°C for 60 s; and a final extension at 72°C for 10 min. In order to remove contaminants such as proteins, divalent cations, unincorporated nucleotides and enzyme inhibitors, PCR products were purified with GRS PCR & gel band purification kit (GRISP, Porto, Portugal) and sent to a specialised research facility (GATC Biotech AG, Constance, Germany) for sequencing. The target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality. Data were analysed using the available BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and FinchTV (Geospiza, Seattle, WA, USA) softwares.

2.6. Real-time PCR

For the real-time PCR amplifications, the reaction mixture of 20 μL included 1 $\times$ of SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA) or 1 $\times$ of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in the case of primers BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2, 240 nM of each primer (Bos-F/Bos-R, 916/916-R, BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2), 150 nM of each probe (Bos-P or 916-P) for *cytb* or 12S rRNA genes, respectively, and 2 μL of DNA extract (40 ng for binary mixtures and 100 ng for meat samples).

Target sequences were amplified using a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: 95°C for 5 min, 50 cycles at 95°C for 15 s and 65°C for 45 s (for primers Bos-F/Bos-R, BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2) or 50 cycles at 95°C for 10 s, 56°C for 10 s and 72°C during 30 s (for primers 916/916-R), with collection of fluorescence signal at the end of each cycle. For melting curve analysis, in real-time PCR assays using EvaGreen dye, the amplicons were denatured at 95°C for 1 min and then annealed at 70°C for 3 min in order to allow the correct annealing of the double strands. These two steps were further followed by melting curve ranging from 70.0 to 95.0°C with temperature increments of 0.2°C every 10 s. The fluorescence data were acquired by the end of each melting temperature. The software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the data from each real-time PCR run. Cycle of quantification (Cq), also known as cycle threshold (Ct) values were calculated using the software at automatic threshold settings. Real-time PCR trials were performed using $n = 4$ replicates in each run.

3. Results

In this study, several sequences targeting different genes from cow's mitochondrial genome and some nuclear genes encoding important milk allergenic proteins were evaluated for their capacity to detect trace amounts of cow's milk in meat products by PCR. Different DNA extraction methods with the optimisation of some steps were assayed, followed by the design of specific primers and their application in qualitative PCR to evaluate their sensitivity and specificity. The most promising primers were tested by real-time PCR using both TaqMan probes or binding dyes, to study their suitability for quantitative purposes by the analysis of the analytical performance parameters.

3.1. DNA extraction

DNA extracts from meat species were obtained with the Wizard method (Mafra et al., 2008), presenting a wide range of yields (195.0–817.7 ng/μL) and adequate purities (1.8–2.1). MPC sample and binary mixtures were extracted using the Wizard method and NucleoSpin food kit (Macherey-Nagel, Düren, Germany) with and without the addition of 2 μL of RNase (2 mg/mL) after the cell lysis step, for comparative purposes. Regarding the first method, the obtained yields varied between 37.0 and 397.6 ng/μL and purities were in the range of 1.8–2.1. NucleoSpin Food kit provided purities between 2.0 and 2.3 and yields of 109.4–888.3 ng/μL without RNase or purities of 1.9–2.0 and yields between 34.2 and 301.0 ng/μL with its use. It was considered that the use of RNase improves the quality of extracts by eliminating PCR inhibitors. All DNA extracts tested positively with universal primers targeting the eukaryotic nuclear 18S rRNA gene (section 2.3), thus confirming the presence of amplifiable DNA and eliminating any potential false negative results in the specific PCR assays.

3.2. Screening gene markers for cow's detection (sensitivity and specificity)

The primers used in this work and the respective optimised qualitative PCR conditions are summarised in Table 1. Firstly, for sensitivity testing, the primers were assayed using 10-fold serial diluted extracts of the model mixture of 10% of MPC in turkey meat (corresponding to 10 ng of cow's milk DNA). The PCR amplifications with all primer sets produced the expected fragments, except for the BLG region. In this case, a fragment of around 500 bp was obtained instead of the expected 129 bp, suggesting the presence of introns probably spliced during the transcription process and, therefore, not reported in the target sequence of bovine mRNA β-lactoglobulin available at NCBI database (accession no. X14712.1). For that reason, the PCR products obtained from the amplification of MPC extracts (10 ng) with primers BLG-F/BLG-R were sequenced. The results showed high-resolution electropherograms, enabling the complete and correct sequencing of 520 bp amplicons. Therefore, a new primer set was designed for this region (BLG-F1/BLG-R1), to specifically amplify fragments of 120 bp (supplementary material, Fig. S1). The PCR assays with all the primer sets showed absolute sensitivities between 1 and 100 pg of bovine DNA, which can be observed in Fig. S2 (supplementary material) presenting some of the obtained results.

In addition to *in silico* analysis, the specificity of the proposed primers for cow's identification was determined experimentally using different meat species commonly used in food products (as described in 2.1 section). Table 2 shows the resumed PCR results of specificity tests, demonstrating a high level of cross-reactivity with some animal species, namely chicken, goat, pork and turkey, which were amplified by the majority primers targeting mitochondrial genes. The specificity results combined with the sensitivity data (supplementary material, Fig. S2), highlighted the primer sets targeting the *cytb* (Bos-F/Bos-R), 12S rRNA (916/916-R), β-casein (BCAS-F1/BCAS-R2) and β-lactoglobulin (BLG-F1/BLG-R1) genes, with the production of fragments of 99 bp (Fig. S2A), 121 bp (Fig. S2C), 106 bp (Fig. S2I) and 120 bp (Fig. S2J) (supplementary material), respectively, as the most promising. Thereafter, the sensitivity of these primers was further evaluated using the binary mixtures of MPC in meat (supplementary material, Fig. S3). Both assays targeting mitochondrial genes presented sensitivities down to 0.01% (100 mg/kg) of MPC in cooked ham and 0.1% (1000 mg/kg) in raw meat (supplementary material, Figs. S3A–S3D), while the assay targeting β-lactoglobulin region was able to amplify 0.05% (500 mg/kg) and 0.01% (100 mg/kg) of MPC in raw turkey meat and cooked ham, respectively (supplementary material, Figs. S3E and S3F). Regarding Bos-F/Bos-R primers, there is a very weak amplification at the level of 0% (supplementary material, lane 9, Figs. S3A and S3B) probably due to the residual cross-reactivity with the matrix. The β-casein PCR assay presented the lowest sensitivity, down to 0.5%

(5000 mg/kg) of MPC in cooked ham and 1% (10,000 mg/kg) of MPC in raw turkey meat mixture (supplementary material, Figs. S3G and S3H).

3.3. Real-time PCR

The primers targeting the mitochondrial (Bos-F/Bos-R and 916/916-R) regions and the milk allergen-encoding genes (BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2) were tested by real-time PCR in order to verify their suitability for a quantitative method. Firstly, cross-reactivity with the critical species, namely chicken, goat, pork and turkey was verified for each system, followed by their application to binary mixtures. The Ct mean values obtained by each run are shown in Table 3. Contrarily to qualitative PCR results (Table 2), where no positive non-target amplification appeared, the real-time PCR assays with EvaGreen dye using primers targeting the allergen-encoding genes presented cross-reactivity with all the tested meat species. Ct mean values ranged from 29.49 ± 0.12 to 35.40 ± 1.75 and from 28.10 ± 0.13 to 32.48 ± 0.33 for β-lactoglobulin and β-casein genes, respectively (Table 3). In addition, the melting temperature obtained in the amplification of β-lactoglobulin gene in MPC sample (84.50 °C) is very similar to the value obtained by the amplification of turkey species (84.40 °C), suggesting the production of a very similar PCR product (data not shown). When both allergen-encoding gene approaches were applied to raw binary mixtures, it was observed a lack of reproducibility between qualitative PCR and real-time PCR data. The clear gradient observed in qualitative PCR results (supplementary material, Figs. S3E and S3G) is not confirmed by the Ct values obtained in real-time PCR (Table 3), showing a difference of only 1.0 (Ct values from 30.82 ± 0.03 to 31.82 ± 0.35) and 1.7 (Ct values from 28.74 ± 0.40 to 30.42 ± 0.34) cycles between the first spiked level (10%) and the last one (0.005%), for β-lactoglobulin and β-casein gene systems, respectively. Moreover, both systems showed amplification for the blank mixture (0%), without containing MPC, at 31.47 ± 0.04 and 30.45 ± 0.23 cycles for the β-lactoglobulin and β-casein systems, respectively. This finding is in accordance with the referred cross-reactivity with turkey species that is present in mixture.

Regarding real-time PCR trials targeting the two mitochondrial genes (*cytb* and 12S rRNA), one TaqMan probe was designed for each (Bos-P and 916-P) in an attempt to eliminate the residual cross-reactivity showed in qualitative PCR results (Table 2) for some meat species. The results were only slightly improved in the case the *cytb* primers, which did not amplify goat, while chicken, turkey and pork showed a delayed amplification. In the case of 12S rRNA primers, turkey was not amplified, though chicken and goat were amplified, but with very high Ct values. Such high Ct values suggest that 12S rRNA system presented the weakest cross-reactivity with goat and chicken compared with the other tested systems (Table 3).

Comparing the application of the two mitochondrial gene systems to raw binary mixtures, the target species is amplified two cycles earlier with *cytb* primers than with 12S rRNA primers in almost all spiked levels, including the sample without the addition of MPC (Table 2), as also obtained by qualitative PCR (supplementary material, Figs. S3A and S3C). Although both mitochondrial systems present consistent Ct values in conformity with the gradient observed in qualitative PCR, only the real-time PCR assay targeting the 12S rRNA gene showed no amplification for the blank matrix (0% level) and amplifications above 38 cycles only for chicken species (Table 3). Thus, Ct values above the threshold of cross-species amplification (> 38) were considered negative. Regarding the amplification of goat (36.91 ± 2.59), it was considered not problematic since this species is not added to this type of meat products due to economic reasons.

As a result, the most promising system targeting the 12S rRNA gene was further assessed with both raw and cooked binary mixtures as a potential candidate for the development of a quantitative method for MPC detection in processed meat products. The assessment of assay performance was according to the general guidelines for method

Table 2

Results obtained by qualitative PCR showing the level of amplification of each meat species tested in cross-reactivity studies.

Primers	Cow	Boar	Duck	Partridge	Hare	Quail	Pheasant	Deer	Rabbit	Chicken	Turkey	Lamb	Goat	Ostrich	Horse	Pork
Bos-F/R	+++	+/-	-	-	+/-	-	-	-	-	+	+	-	+	-	-	+
Bcyt-F/R	+++	-	-	-	-	-	-	-	-	+/-	+/-	-	+++	-	-	++
Beef-F/R	+++	-	-	-	-	-	-	-	-	++	-	-	++	-	-	++
B-F1/R1	+++	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+++
B-F2/R2	+++	-	-	-	-	-	-	-	-	+/-	-	-	+	-	-	+++
916/1171	+++	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	+/-
916/916-R	+++	-	-	-	-	-	-	-	-	-	-	-	++	-	-	+/-
916-F/R	+++	-	-	-	-	-	-	-	-	-	+	-	+++	-	-	+/-
916-F1/R	+++	-	-	-	-	-	-	-	-	-	+/-	-	+++	-	-	+/-
B12S-F/R	+++	-	-	-	-	-	-	-	-	+/-	++	-	++	-	-	++
mit-F/R	+++	-	-	-	-	-	-	-	-	+/-	-	-	+++	-	-	++
mit-F2/R	+++	-	-	-	-	-	-	-	-	+/-	-	-	+	-	-	++
BCOI-F/R	+++	-	-	-	-	-	-	-	-	+/-	+	-	+	-	-	++
Bmt-F1/R	+++	-	-	-	+/-	-	-	-	-	+/-	+	-	++	-	-	++
Bmt-F2/R	+++	-	-	-	-	-	-	-	-	+	+	-	+++	-	-	+
tRD-F/R	+++	-	-	-	-	-	-	-	-	-	+	-	+++	-	-	++
ND2-F/R	+++	-	-	-	-	-	-	-	-	+/-	++	-	++	-	-	++
ND4-F/R	+++	-	-	-	-	-	-	-	-	++	+	-	++	-	-	++
ND4L-F/R	+++	-	-	-	-	-	-	-	-	-	+	-	++	-	-	++
BCAS-F1/R2	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BLG-F1/R1	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+ + +) very strong positive amplification; (+ +) strong positive amplification; (+) weak amplification; (+ -) very weak amplification; (-) negative amplification.

Table 3

Real-time PCR results for the cross-reactivity test of each system with the meat species, chicken, turkey, goat and pork and with the binary mixtures of raw turkey meat with MPC-85 ranging from 0% to 10%.

Target	Primers					
	916/916-R		Bos-F/Bos-R		BLG-F1/BLG-R1	
	Ct $\pm$ SD ^a					
	Raw		Cooked		Raw	
Meat species	Chicken	38.79 $\pm$ 0.37 (3/3) ^b			34.59 $\pm$ 1.18 (3/3)	35.40 $\pm$ 1.75 (3/3)
	Turkey	NA			38.60 $\pm$ 0.33 (2/3)	32.71 $\pm$ 1.15 (3/3)
	Goat	36.91 $\pm$ 0.59 (3/3)			NA	29.94 $\pm$ 0.34 (3/3)
	Pork	NA			35.45 $\pm$ 0.97 (2/3)	29.49 $\pm$ 0.12 (3/3)
Milk protein concentrate (MPC)		22.63 $\pm$ 0.53 (3/3)			21.16 $\pm$ 0.07 (3/3)	22.85 $\pm$ 0.06 (3/3)
Spiked level of MPC in raw meat (%)	0	NA	NA		37.44 $\pm$ 0.00 (1/4)	31.47 $\pm$ 0.04 (4/4)
	0.005	39.85 $\pm$ 0.51 (4/4)	39.86 $\pm$ 1.00 (4/4)		38.71 $\pm$ 0.76 (4/4)	31.82 $\pm$ 0.35 (4/4)
	0.01	39.35 $\pm$ 0.39 (4/4)	38.67 $\pm$ 0.32 (4/4)		36.55 $\pm$ 0.33 (4/4)	31.91 $\pm$ 0.39 (4/4)
	0.05	37.51 $\pm$ 0.40 (4/4)	36.87 $\pm$ 0.75 (4/4)		35.25 $\pm$ 0.74 (4/4)	31.90 $\pm$ 0.14 (4/4)
	0.1	36.54 $\pm$ 0.35 (4/4)	36.55 $\pm$ 0.12 (4/4)		34.47 $\pm$ 0.59 (4/4)	32.15 $\pm$ 0.07 (4/4)
	0.5	34.61 $\pm$ 0.17 (4/4)	33.53 $\pm$ 0.14 (4/4)		32.63 $\pm$ 0.25 (4/4)	32.03 $\pm$ 0.09 (4/4)
	1	33.26 $\pm$ 0.16 (4/4)	32.55 $\pm$ 0.28 (4/4)		31.28 $\pm$ 0.27 (4/4)	31.97 $\pm$ 0.03 (4/4)
	5	30.26 $\pm$ 0.09 (4/4)	29.19 $\pm$ 0.12 (4/4)		28.58 $\pm$ 0.08 (4/4)	31.75 $\pm$ 0.03 (4/4)
	10	28.47 $\pm$ 0.17 (4/4)	27.82 $\pm$ 0.07 (4/4)		27.31 $\pm$ 0.19 (4/4)	30.82 $\pm$ 0.03 (4/4)

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD).^b Positive amplifications/total replicates; NA - no amplification.

development described by Bustin et al. (2009) and ENGL (2015), whose criteria established for real-time PCR assays were carefully considered: the limit of detection (LOD) should be defined as the lowest concentration at which 95% of the replicates are detected, the PCR efficiency should range between 90 and 110%, the slope should be within -3.6 and -3.1 and the correlation coefficient (R^2) above 0.98. Fig. 1 presents the amplification curves and respective calibration curves for the real-time PCR 12S rRNA system applied to both raw and cooked model mixtures. The calibration curves presented slopes of -3.504 and -3.647 , R^2 of 0.976 and PCR efficiency of 92.9% and 88.0% for raw and cooked hams, respectively (Fig. 1), which were within or very close to the adequate performance criteria parameters. Both calibration curves enabled a dynamic range of 4 orders of magnitude, covering the amplification from 0.005% (w/w) (50 mg/kg) of MPC in raw and cooked hams (Table 3). However, considering that amplifications above 38 cycles should not be accounted due to non-target amplification, the relative LOD should be defined as 0.05% (w/w) (500 mg/kg) for both

raw and cooked binary mixtures.

4. Discussion

Some food proteins are used as technological aids in the preparation of uncounted food products providing functional and nutritional properties to the final product. However, in most cases, these type of aids are considered important allergens, such as celery, soy and cow's milk proteins (Spychaj et al., 2018). They are often present only at trace amounts or masked by the food matrix, which usually makes their detection very difficult to achieve, representing a constant threat to allergic individuals. In the specific case of meat products, such as hams and sausages, milk protein concentrates and isolates are frequently added (Uluko et al., 2016). Presently, there are numerous technical possibilities for the detection of allergens in foodstuffs, targeting either the allergen itself (MS spectrometry and immunochemical methods) or an indirect molecular marker (DNA-based techniques) that indicates the

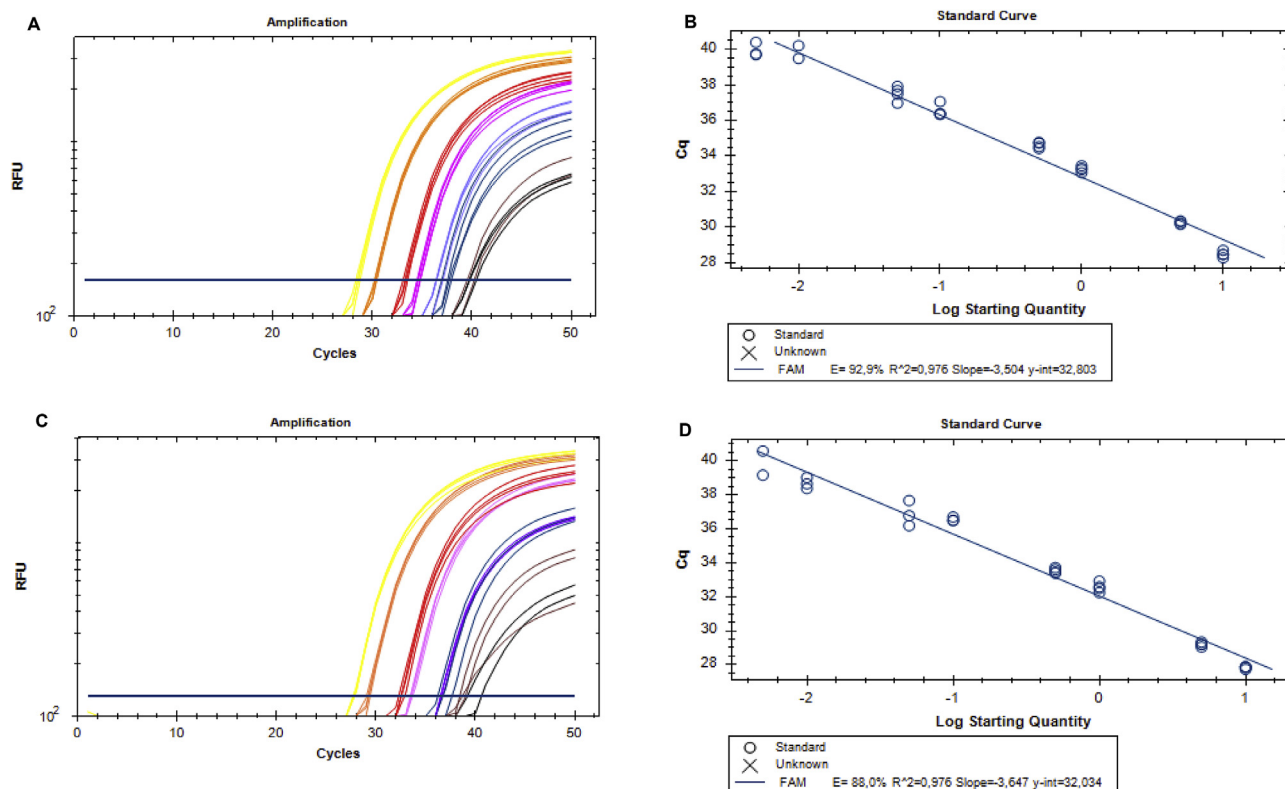


Fig. 1. Amplification curves (A, C) and respective calibration curves (B, D) obtained by real-time PCR targeting the bovine 12S rRNA mitochondrial gene using the binary mixtures of cow's milk protein concentrate (MPC) in turkey raw meat (A, B) and in cooked hams (C, D) (10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005% (w/w)) ($n = 4$).

presence of the offending food. The selection of the target/biomolecule becomes critical since it will greatly affect the sensitivity, the reliability and the quantification capacity of the technique (Costa et al., 2017a). Meat products are submitted to different thermal treatments during their preparation (autoclaving and cooking procedures, such as roasting, baking, grilling or boiling), which can induce alterations in the properties of the food components, such as hydrolysis and structural changes of proteins (Costa, Amaral, Grazina, Oliveira, & Mafra, 2017b). In these cases, techniques targeting DNA markers are advantageous alternatives for the analysis of highly processed foods. DNA targets might be genes that encode allergenic proteins or other specific sequences, thus being considered as indirect markers of the presence of an allergenic ingredient (Villa et al., 2018).

In order to develop a sensitive and accurate method to detect and quantify milk ingredients in meat products, a complete screening and evaluation of several mitochondrial and nuclear genes as candidate markers for cow detection were performed to be used as specific and indirect targets for milk allergen detection. For this purpose, binary mixtures of MPC in turkey meat, simulating the production of cooked ham were prepared and submitted to a thermal treatment at 67 °C during 5 h in the oven, accounting with the interference of food matrix and processing to assess the most promising genes for method development. In order to achieve better sensitivity results, multi-copy genes (mitochondrial genes) were mostly used. However, it was observed a strong cross-reactivity with some meat species using almost of the designed primers due to the highly conserved nucleotide sequences of these genes in different species (Holzhauser, Stephan, & Vieths, 2006). Since single-copy genes are reported to be more specific (Holzhauser et al., 2006), they were also tested in the present work. Based on the best sensitivity and specificity results obtained by qualitative PCR, four primer pairs targeting two mitochondrial (*cytb* and 12S rRNA) and two nuclear (β -lactoglobulin and β -casein) genes were chosen from a total of 22 (Table 1), mostly designed in this work and further tested in

binary mixtures. Generally, it was observed a better relative sensitivity in cooked binary mixtures than in raw, possibly due to a strong reduction in water content during thermal treatment, thus causing an improvement in DNA extraction and PCR efficiency. Additionally, raw meat has high enzymatic activity, which is greatly reduced during mild food processing (oven cooked at 67 °C for 5 h) by inactivation phenomena and water loss, thus contributing to stabilise total DNA, including the target one. Although thermal processing is known to contribute to shearing and degradation of DNA of high molecular mass (Fernandes, Oliveira, & Mafra, 2013), other factors such as enzymatic activity together with high water content and the presence of PCR inhibitors (Costa et al., 2013; 2017b) are also determinant for the success of PCR amplification. The relative sensitivity in qualitative PCR was down to 0.01% (100 mg/kg) of MPC in cooked ham with primers targeting both mitochondrial and β -lactoglobulin genes (supplementary material, Fig. S3).

To improve specificity of the two selected multi-copy regions, specific TaqMan probes were designed on the *cytb* and 12S rRNA sequences in real-time PCR assays using binary mixtures. On the other hand, allergen-encoding sequences were amplified with a third generation binding dye (EvaGreen), which offers enhanced fluorescence, increased sensitivity and excellent stability (Wang, Chen, & Xu, 2006). However, these trials were not consistent with qualitative PCR results probably due to the enhanced real-time PCR amplification conditions, highlighting reactivity with non-target species (chicken, turkey, goat and pork) (Table 3) and disabling their utility. The use of mitochondrial genes combined with specific TaqMan probes seemed to provide better specificity results, comparing with nuclear genes, suggesting only minor or no cross-reactivity for chicken, turkey, goat and pork. The 12S rRNA system was considered as the best choice for the development of a quantitative real-time PCR method since turkey meat was not amplified, while the other three cross-reactive meat species exhibited delayed amplification (above 38 cycles). The obtained calibration curves using

both raw and processed binary mixtures presented, generally acceptable PCR performance parameters, achieving a relative LOD of 0.05% (500 mg/kg). The calibration curve for the thermally treated binary mixtures displayed a PCR efficiency slightly lower (88%) than the acceptance criteria generally recommended (90–110%). However, since it is a complex processed food matrix, it is acceptable to be within the range of 75–110% (ENGL, 2015).

The majority of works using DNA-based methods detecting cow in food products focuses on dairy product authentication. Some authors using multi-copy genes reported the problem of cross-reactivity with other meat species. In those cases, Ct values above the threshold of cross-species DNA amplification should be considered negative (Drummond et al., 2013; Zhang, Fowler, Scott, Lawson, & Slater, 2007). With such a high level of unspecific amplifications, LOD are never below 0.1% of cow DNA (Kalogianni, 2018). Using the *cytb* region, Cottenet, Blancpain, and Golay (2011), and Drummond et al. (2013) were able to detect 1% of bovine DNA, while Lopparelli, Cardazzo, Balzan, Giaccone, and Novelli (2007) and Di Domenico, Di Giuseppe, Wicochea Rodríguez, and Cammà (2017) reached a sensitivity of 0.5% and 0.1%, respectively. Rentsch et al. (2013) detected 0.2% of cow DNA in cheeses using the bovine beta actin nuclear gene. A method developed by Liao, Liu, Ku, Liu, and Huang (2017) targeting the mitochondrial 12S rRNA gene was able to detect 0.1% of cow's milk in goat milk powders. However, in the field of food allergen detection, methods need to be more sensitive and able to detect trace amounts of the offending food. Xiao et al. (2016) performed a real-time PCR with a TaqMan minor groove binder probe to detect the α -lactalbumin-encoding gene, obtaining a sensitivity down to 0.05 ng of bovine DNA. However, they only used 10-fold serial dilutions of bovine DNA isolated from cow milk as template, without attempting any reference mixture to provide a realistic and practical level to estimate cow's milk content as an ingredient. Köppel et al. (2010, 2012) were able to detect milk as an allergen in processed foods, but without quantitative results, presenting a sensitivity of 0.64 μ g/mL of bovine DNA.

In the present work, we have realised that the development of a DNA-based method to detect milk as a potential allergenic ingredient, complying with high levels sensitivity and specificity, was a very challenging task, requiring concerted *in silico* and experimental efforts. Nevertheless, with the real-time PCR approach targeting the 12S rRNA gene, we could reach an acceptable compromise of sensitivity/specificity. The assay was the most specific since it provided only minor reactivity with two species at delayed amplification cycles (> 36), linearity in the range of 10–0.005% of cow's MPC in raw meat and cooked ham, but a LOD of 0.05%, considering cross-reactivity data. The preliminary real-time PCR assay provided acceptable performance parameters, being the starting point to propose a novel method to quantify trace amounts of milk ingredients as potential allergens in complex meat products, which should be further validated and applied.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2019.106823>.

Declaration of interest

The authors declare no conflict of interests.

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Supplementary material

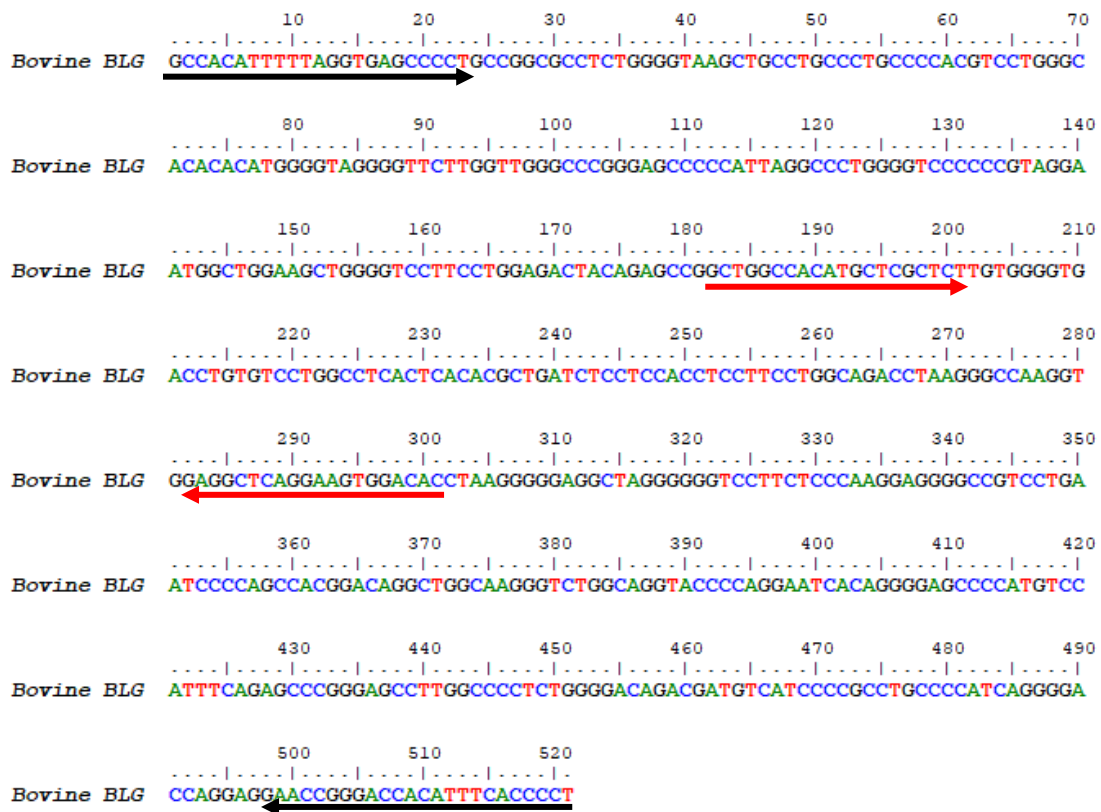


Fig. S1. Sequencing product of bovine BLG region (520 bp). Black arrows represent sequencing BLG-F/BLG-R primers and red arrows represent the new BLG-F1/BLG-R1 primers designed to amplify a fragment of 120 bp.

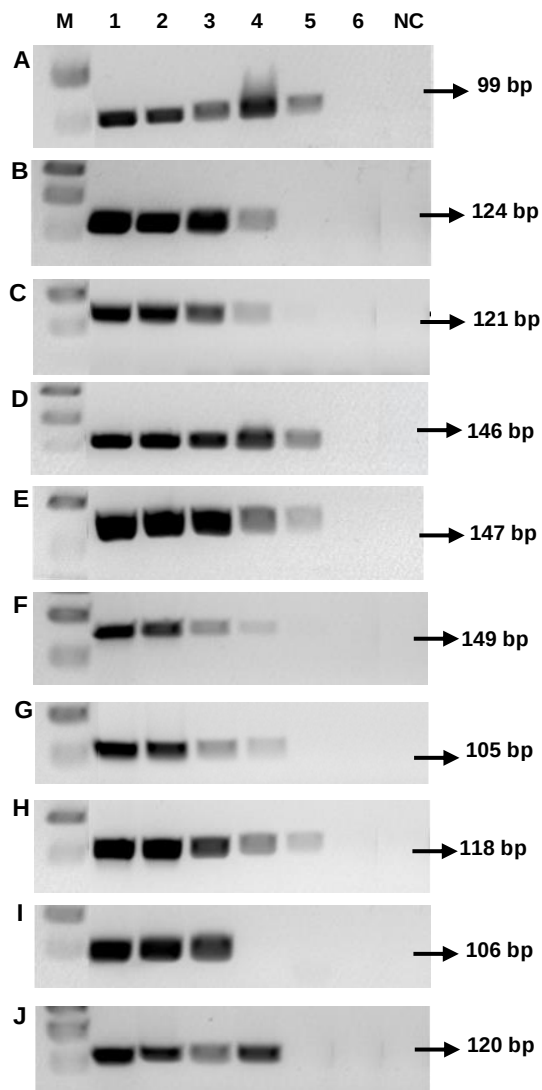


Fig. S2. Agarose gel electrophoresis of PCR products obtained using some of the primers designed in this work to specifically amplify cow DNA. (A) Bos-F/Bos-R and B-F1/B-R1 (B) primers targeting *cytb* gene; (C) 916/916-R primers targeting 12S rRNA gene; (D) Bmt-F1/Bmt-R1 primers targeting tRNA-Lys/ATP synthase F0 subunit 8 genes; (E) tRD-F/tRD-R primers targeting tRNA-Pro/D-loop genes; (F) ND2-F/ND2-R primers targeting NADH dehydrogenase subunit 2 gene; (G) ND4-F/ND4-R primers targeting NADH dehydrogenase subunit 4 gene; (H) ND4L-F/ND4L-R primers targeting NADH dehydrogenase subunit 4L gene; (I) BCAS-F1/BCAS-R2 primers targeting β -casein gene; (J) BLG-F1/BLG-R1 primers targeting β -lactoglobulin gene; M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany); lane 1, 10000 pg; lane 2, 1000 pg; lane 3, 100 pg; lane 4, 10 pg; lane 5, 1 pg; lane 6, 0.1 pg; NC, negative control.

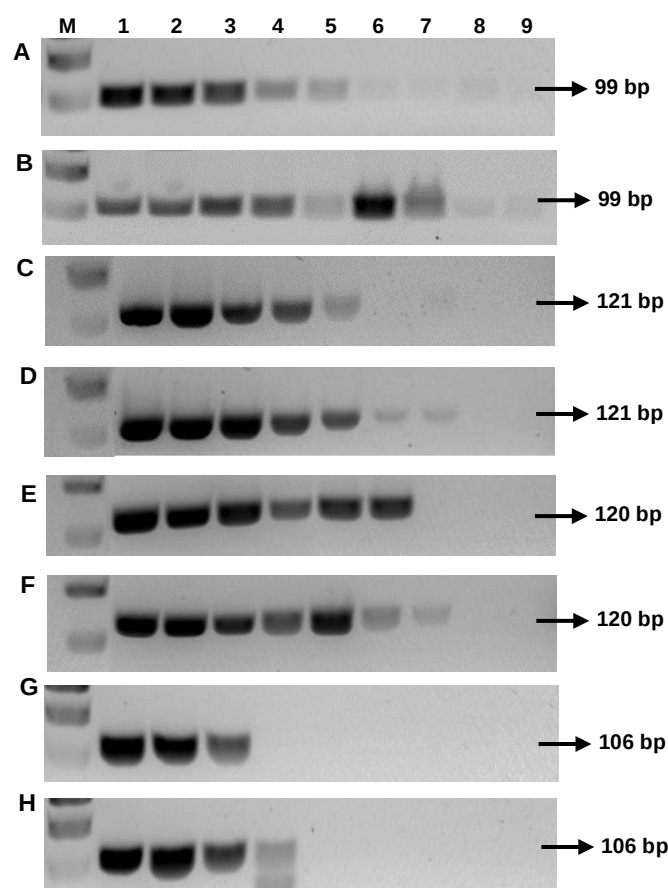


Fig. S3. Agarose gel electrophoresis of PCR products targeting *cytb* (A, B), 12S rRNA (C, D), β -lactoglobulin (E, F) and β -casein (G, H) genes using DNA extracts of binary mixtures of MPC in raw (A, C, E, G) meat and in cooked hams (B, D, F, H) from 10% to 0% (w/w). M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany); lane 1, 10%; lane 2, 5%; lane 3, 1%; lane 4, 0.5%; lane 5, 0.1%; lane 6, 0.05%; lane 7, 0.01%; lane 8, 0.005%; lane 9, 0%.