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Comparison of three methods of DNA extraction from cold-smoked salmon and impact of physical treatments

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Abstract: Aims: To compare three bacterial DNA extraction procedures on cold-smoked salmon (CSS) and assess the impact on their efficiency of two physical treatments of the food matrix, ionizing irradiation and freezing.

Methods and Results: As molecular methods for bacterial detection have become an important analytical tool, we compared bacterial DNA extraction procedures on CSS. Working with frozen and irradiated CSS, we obtained negative responses from samples known to be highly contaminated. Thus, we decided to study the impact of these two physical treatments on bacterial DNA extraction procedures. The efficiency of bacterial DNA extraction directly from the fish matrix suspension was measured by an rpoB PCR-based reaction. The results demonstrated that the DNeasy® tissue extraction kit (Qiagen, Courtaboeuf, France) was the most efficient and reproducible method. We also showed that freezing and ionizing irradiation have a negative impact on DNA extraction. This was found probably not to be due to inhibition as the PCR reaction remained negative after adding BSA to the PCR mix reaction.

Conclusions: The extraction kit was the most efficient method. Physical treatments were shown to hamper bacterial DNA extraction.

Significance and Impact of the Study: Attention must be paid to molecular bacterial detection on food products subject to freezing or to ionizing irradiation.

Keywords: cold-smoked salmon, DNA extraction, freezing, ionizing irradiation, PCR, rpoB gene.

INTRODUCTION

Molecular methods, such as PCR-based techniques, are emerging as ways to study microbial diversity in environmental samples as well as in food products without cultivation. To overcome the disadvantages of culture-dependent methods, the use of PCR to detect bacteria in food has been developed in recent years. However, the extraction of bacterial DNA from heterogeneous food material is often a hurdle. Differences in cell wall structure and in adhesion properties of micro-organisms together with physical, chemical and biological food characteristics affect this extraction. In fish products, some authors have reported bacterial DNA extraction methods from an enriched broth of the crushed flesh of different seafoods (Agersborg *et al*., 1997) or cold-smoked freshwater fish (Gonzalez-Rodriguez *et al*.,2002) but the aim of these studies was to validate the presence or absence of food-borne pathogenic bacteria. To study bacterial diversity, pre-enrichment cannot be used because it could lead to bias due to unequal development of different bacterial species in the pre-enrichment broth.

The use of appropriate DNA extraction procedures directly on crude samples is critical for successful and valid PCR studies on clinical, environmental or food samples. Many methods of extracting bacterial DNA directly from the crushed mother solution, including rapid methods and commercial kits, have been compared (McOrist *et al*., 2002; Anderson and Lebepe-Mazur, 2003; Li *et al*., 2003; Sharma *et al*., 2003). Though the effectiveness of some commercial kits has often been demonstrated, no standard or universal method has been shown to be the most efficient.

In cold-smoked salmon (CSS), microbial diversity has been studied using culture-independent approach (Cambon-Bonavita et al., 2001; Rachman et al., 2003). In these studies, and in accordance with statements made by Giraffa and Neviani (2001) in their review, DNA extraction has been highlighted as one of the limitations of the culture-independent methods.

Thus, the aim of our work was to define the best protocol to extract bacterial DNA from contaminated CSS by comparing three different extraction methods. As there is no direct way to measure specifically bacterial DNA after extraction, bacterial DNA from CSS was evaluated as a template by determining the efficiency of PCR amplification performed on the total DNA extracted. Primers targeting the *rpoB* gene were employed. The *rpoB* gene has been estimated as a suitable biomarker of microbial biodiversity (Dahlöf *et al*., 2000 ; Giacomazzi *et al*. 2004).

For inoculation trials, freezing and ionising irradiation are generally used in our laboratory to produce a sterile CSS experimental model (Joffraud et al. 1998). A poor recovery of bacterial DNA from frozen and irradiated CSS has previously been obtained. It is well known that freezing can degrade proteins and lipids in food products and has an impact on microbial development (Genot, 2000) but little information is available about its impact on DNA. The effect of freezing on DNA extraction has previously been reported for products such as bovine thymus (Kreader, 1996) and urine (Vu et al., 1999) but not for bacterial DNA isolated from a food matrix. Thus, an evaluation of the effect of freezing and ionising irradiation of CSS on bacterial DNA retrieval was also performed in our study.

MATERIALS AND METHODS

Strains, media and artificial contamination

Strains used for artificial contamination of CSS were a Gram positive *Carnobacterium piscicola* SF668 and a Gram negative *Photobacterium phosphoreum* SF714 previously isolated from CSS (Leroi et al., 1998). Cultures were grown twice at 20°C on Brain Heart Infusion (BHI, Biokar, France) before being diluted in chilled physiological saline water (8.5 g l^{-1} NaCl and 1 g l^{-1} tryptone Biokar). Slices of CSS (40 g) were inoculated with 2 % (v/w) of appropriate culture dilution. Inoculation was performed by spreading drops onto the surface of the slices with a Pasteur pipette. Slices were then vacuum-packed and left for 12 h at 4°C to allow bacterial adhesion before DNA extraction.

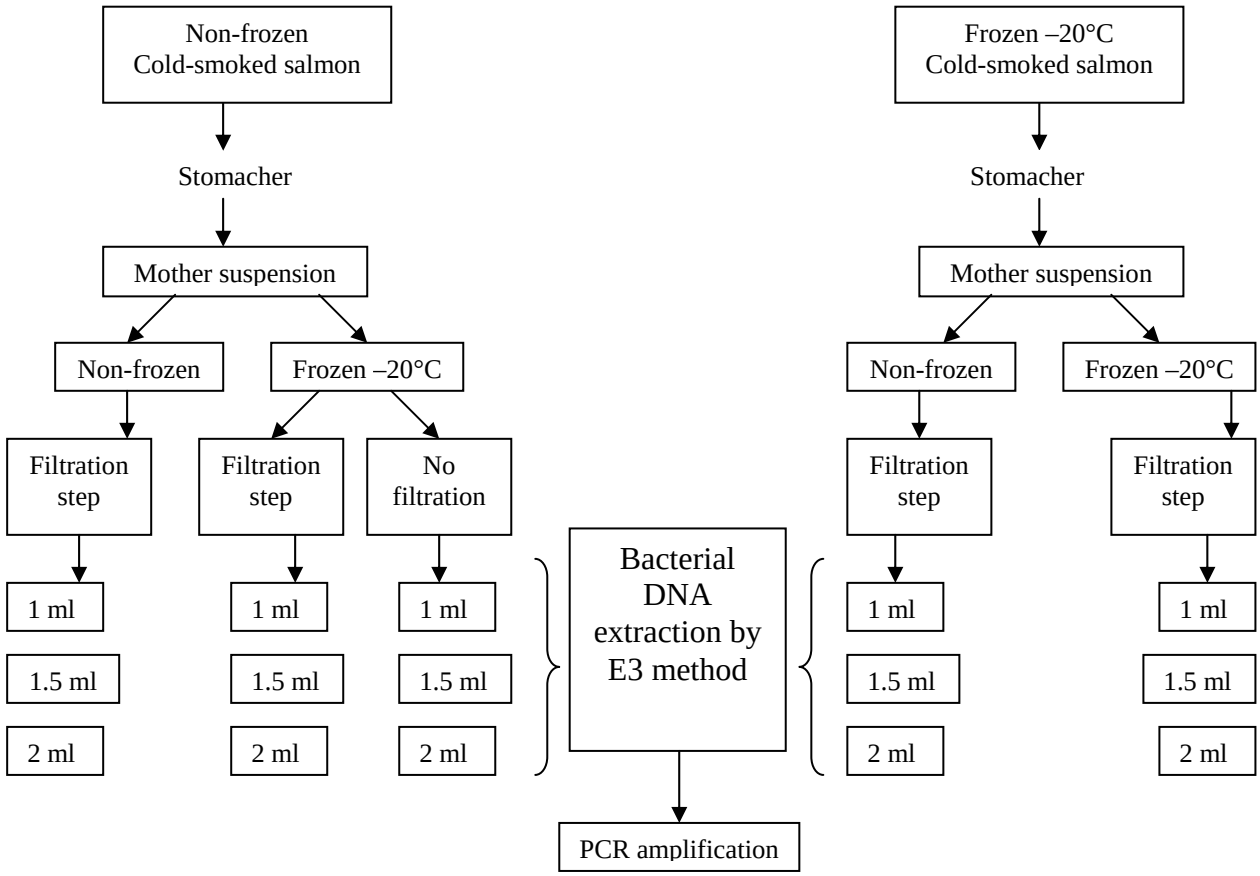
Comparison of efficiency and detection limits of bacterial DNA extraction procedures

Sliced vacuum-packed CSS was purchased from a producer (A) providing products of high hygienic quality (< 20 CFU g^{-1}). It was used to estimate bacterial DNA extraction using the three methods described below. Extraction was performed just after production and after six weeks of vacuum storage at 4°C to appreciate the extraction of a naturally spoiled product. Next, slices were sterilised in freezing conditions by an electron beam ionisation technique (SPI, Berric, France) at 2 kGy (Joffraud al., 1998). Thawed slices were then artificially inoculated with around 10, 10^3 and 10^6 bacteria per gram of flesh in order to study detection limits of bacteria in CSS. Some slices were inoculated with *C. piscicola* SF668 and others with a mixture of *C. piscicola* SF668 and *P. phosphoreum* SF714.

Impact of freezing, the filtration step and volume on bacterial DNA extraction

Sliced vacuum-packed CSS was purchased from a supermarket. CSS was stored at 20°C for 2 d and then at 8°C for 3 d in order to allow rapid bacterial development and a sufficient level of contamination. The impact of freezing (both of CSS and of the mother solution) on bacterial DNA extraction was studied using the protocol described in Fig. 1. Some slices were directly used for DNA extraction and others after freezing at -20°C. Respective mother suspensions were also frozen at -20°C or not. A filtration step was tested (see DNA extraction) and extraction was performed on 1, 1.5 or 2 ml of mother solution with the E3 technique described below.

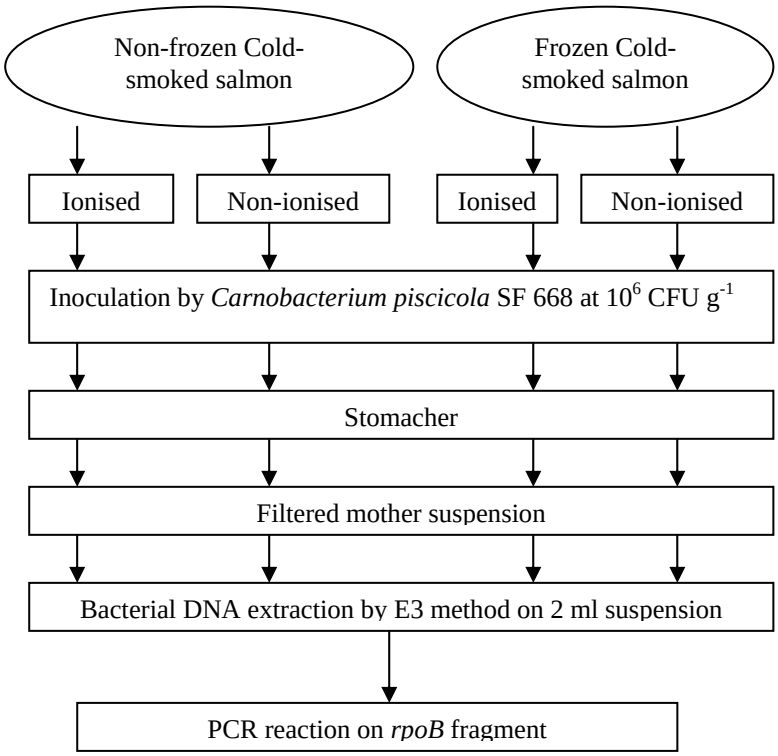
Fig. 1 Protocol designed to study the impact of freezing inoculated cold-smoked salmon and/or the mother suspension on the extraction of bacterial DNA.



Impact of ionising irradiation on bacterial DNA extraction

Sliced vacuum-packed CSS was purchased from the producer mentioned above (A). The impact of irradiation on bacterial DNA extraction was studied using the protocol described in Fig. 2. Some slices were irradiated fresh or in freezing conditions (-20°C). Other non-irradiated slices were used fresh or thawed after the freezing period. All slices were inoculated with *C. piscicola* SF668 at the final concentration of 10^6 CFU g^{-1}. Extraction was performed with the E3 technique described below.

Fig. 2 Protocol designed to study the impact of ionising irradiation of cold-smoked salmon combined with freezing on the extraction of bacterial DNA.



Bacterial DNA extraction

Three samples were processed for each experiment. 30 g of sample was homogenised in 120 ml of chilled physiological saline water using a stomacher (Lab. Blender, London, UK) for 2 min. Bacteria were enumerated on Plate Count Agar (PCA, Biokar, France) by the classical spread plate technique.

A 5 ml aliquot (or 1 ml for the E3 method described below) of the homogenised CSS flesh was filtered under vacuum on an 11 µm nylon filter Scrynel Nylon NYHC (Labosi, France). The filtered suspension was then centrifuged at 10 000 g for 10 min at 4°C. The supernatant was removed and the pellet containing bacteria, but also salmon cells, was treated in three different ways to induce lysis:

Extraction 1 (E1): Direct DNA extraction was performed from the pellet by first using a chemical cell lysis treatment (proteinase K, SDS and Sarkosyl). This was then completed by a physical treatment (microwave heating) to ensure the release of DNA from more resistant bacteria, such as those belonging to the Gram positive group. Thus the pellet was resuspended in 5 ml of lysis buffer (10 mM Tris-HCl, 50 mM Na_2EDTA, 100 mM NaCl, pH 8) containing 1% SDS (Sigma, St Quentin Fallavier, France), 1% Sarkosyl (Sigma, France) and 0.4 mg ml^{-1} of proteinase K (Qiagen, Courtaboeuf, France). The mixture was incubated for 3 h at 45°C under agitation. All centrifugation steps were carried out at 10 000 g, 10 min, 4°C. Lysate was centrifuged and the supernatant was collected and stored on ice until the phenol/chloroform/isoamyl alcohol extraction step to prevent DNA degradation that might occur during the physical cell lysis treatment. The pellet was frozen at –80°C and thawed for 30 s in a 400 W microwave oven three times successively and the supernatant added to the corresponding supernatant of the first lysis treatment kept on ice. Then DNA was extracted

from this supernatant by adding an equal volume of phenol/ chloroform /isoamyl alcohol mixture (25:24:1 v/v/v) (Acros Organics, Noisy-le-Grand, France). The tube was briefly vortexed to obtain an emulsion then centrifuged. The aqueous layer was collected. Traces of phenol were removed by adding an equal volume of chloroform. After centrifugation, the top layer was collected and DNA was precipitated overnight at –20°C with two volumes of cold isopropanol. The pellet of crude DNA was obtained by centrifugation for 20 min and was washed with 1 ml of 70 % cold ethanol. It was air dried and resuspended in 200 µl of TE1X buffer (10 mM Tris-Cl, 1 mM; Na_2EDTA, pH 8).
Extraction 2 (E2): The Pitcher method (Pitcher et al, 1989), modified by Björkroth and Korkeala (1996), was applied. Lysis was performed by adding lysozyme and mutanolysine to the pellet obtained from centrifugation of the filtered suspension of the salmon flesh.
Extraction 3 (E3): A commercial Qiagen DNeasy tissue kit (Qiagen, France) was used. DNA was extracted on 1 ml of the mother solution according to the manufacturer's instructions for Gram positive bacteria.
5 µl DNA from each extraction method was used for PCR amplification.

PCR amplification

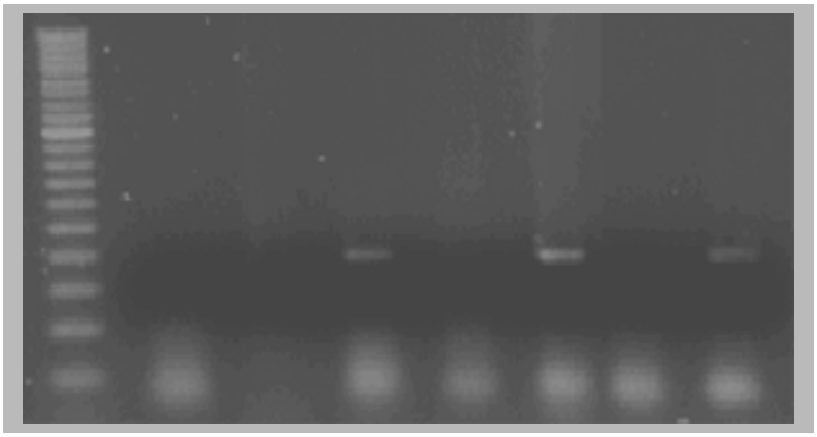
Degenerated primers *rpoB*-1675 and *rpoB*-2063 (Giacomazzi, 2002; Gravelat, 2002) were used for the PCR reaction. The PCR mixture (25 µl) contained as final concentrations: 200 µmol l^{-1} of deoxynucleotide triphosphate mix (Eppendorf, Le Pecq, France), 1 X TaqMaster buffer, 1X Taq buffer with $MgCl_2$ (1.5 mmol l^{-1} Mg^{2+}), 0.2 µmol l^{-1} of each primer *rpoB* (Proligo, Paris, France). The mix was kept frozen in 20 µl aliquots. 0.5 U of MasterTAQ polymerase (Eppendorf) and 5 µl of template DNA previously extracted were added to the thawed mix before PCR amplification was performed on a PCR*sprint* thermocycler (Hybaid, Middlesex, UK). PCR was carried out using the following protocol; initial denaturation (1 min, 94°C), followed by 40 cycles of denaturation (94°C for 20 s), primer annealing (58.5°C for 30 s) and extension (72°C for 45 s). A final extension at 72°C for 10 min was performed. The size and the amount of amplified DNA were verified by electrophoresis on 1 % (w/v) agarose gel (Eurogentec, Seraing, Belgium) containing ethidium bromide 8 10^{-4} mg/ml as final concentration . Gel was photographed using the image viewer ImageMaster VDS-CL (Amersham Pharmacia Biotech, Orsay, France).

RESULTS

Comparison of efficiency and detection limits of bacterial DNA extraction procedures

PCR amplification of the *rpoB* fragment on DNA extracted from the non-inoculated CSS (control) was compared with amplification of bacterial DNA extracted from the same CSS inoculated with *C. piscicola* SF668 as a positive reference. Colony-forming units (CFU) of endogenous and inoculated bacteria were estimated on PCA plates. No colony was detected in the control (detection threshold: 50 CFU g^{-1}) while total count was $5x10^2$ CFU g^{-1} in the inoculated sample. Amplification of the *rpoB* fragment on bacterial DNA extracted from the inoculated sample with each technique was satisfactory (Fig. 3). With E2, the PCR product showed a higher intensity compared to E1 and E3. No PCR product could be detected on the control in accordance with the plate count. The same protocols were also applied to the same CSS (not inoculated) stored for six weeks at 4°C whose plate count was 10^6 CFU g^{-1}. PCR products showed a good intensity for the E2 and E3 methods but a very weak intensity for E1 (data not shown).

Fig. 3 Comparison of bacterial DNA extraction procedures on inoculated and non-inoculated fresh cold-smoked salmon. M: 5 µl of Mass Ruler^{TM} DNA ladder low range (MBI Fermentas, Lithuania); T-: negative control; lanes 1, 2, 3: PCR products of non-inoculated cold-smoked salmon extracted respectively by the E1, E2 or E3 method; lanes 1', 2', 3': PCR products of cold-smoked salmon inoculated by 5 10^2 CFU g^{-1} of *C. piscicola* extracted respectively by the E1, E2 or E3 method.



In a second step, the bacterial detection limit allowing an amplification response was determined working with irradiated CSS inoculated with different levels of bacteria. The concentration of *C. piscicola* was estimated at 10, 1.7 x 10^2, and 1.7 x 10^5 CFU g^{-1}. Due to the detection limit of the spread plate count method (50 CFU g^{-1}), the count at 10 CFU g^{-1} was estimated from the inoculation solution concentration but not directly verified in the flesh. For the mixture of two species, concentrations of *C. piscicola* / *P. phosphoreum* were respectively 10 / 10, 5.8 x 10^2 / 4.5 x 10^2 and 3.8 x 10^5 / 3.9 x 10^5 CFU g^{-1}. The three DNA extraction methods were applied and compared. As shown in Fig. 4, only the E3 method led to the amplification of the *rpoB* fragment. The signal was very weak compared to that (not shown) obtained for DNA extracted from indigenous bacteria of a fresh (not irradiated) naturally spoiled CSS thus the detection limit was difficult to establish. However, contrary to CSS inoculated with *C. piscicola* alone, a signal was observed even at the lowest level of inoculation (10 CFU g^{-1}) for the mixture of bacteria whose PCR amplification was better, probably because of the Gram positive or Gram negative nature of the inoculated bacteria. The results of these experiments are summarised in Table 1.

Table 1: Comparison of the efficiency of the three DNA extraction methods.

	DNA extraction method		
Samples	E1	E2	E3
Non-inoculated CSS (Fig. 3)	-	-	-
Inoculated CSS (Fig. 3)	+	++	+
Irradiated and inoculated CSS (Fig. 4):			
C. piscicola 10 CFU g^{-1}	-	-	+/-
C. piscicola 1.7 10^2 CFU g^{-1}	-	-	+/-
C. piscicola 1.7 10^5 CFU g^{-1}	-	-	+
C. piscicola 10 CFU g^{-1}+ *P. phosphoreum* 10 CFU g^{-1}	-	-	+
C. piscicola 5.8 10^2 CFU g^{-1}+ *P. phosphoreum* 4.5 10^2 CFU g^{-1}	-	-	+
C. piscicola 3.8 10^5 CFU g^{-1}+ *P. phosphoreum* 3.9 10^5 CFU g^{-1}	-	-	+

Fig. 4 Comparison of bacterial DNA extraction procedures on irradiated and inoculated cold-smoked salmon. M: 5 µl of Mass Ruler^{TM} DNA ladder low range (MBI Fermentas, Lithuania); *Cp*: positive control, DNA of the reference type strain of *Carnobacterium piscicola*; T-: negative control; lane 1: *C. piscicola* SF 668 10 CFU g^{-1}; lane 1': *C. piscicola* SF 668 1.7 10^2 CFU g^{-1}; lane 1'': *C. piscicola* SF668 1.68 10^5 CFU g^{-1}; lane 2: *C. piscicola* SF 668 10 CFU g^{-1} and *P. phosphoreum* 10 CFU g^{-1}; lane 2': *C. piscicola* SF 668 5.8 10^2 CFU g^{-1} and *P. phosphoreum* $^{4.5}$ 10^2 CFU g^{-1}; lane 2'': *C. piscicola* SF 668 3.8 10^5 CFU g^{-1} and *P. phosphoreum* 3.8 10^5 CFU g^{-1}. E1, E2, E3: extraction methods 1, 2, or 3 respectively.

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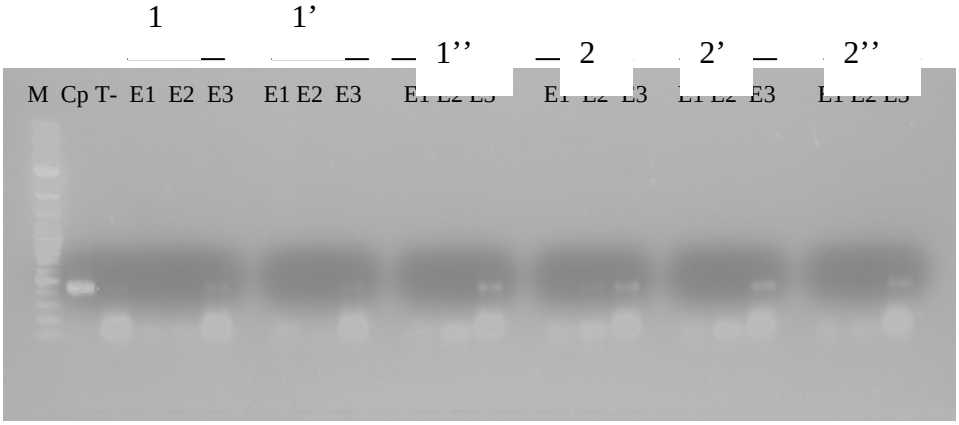

In the second part of this study, we investigated why the signal was lower than in the experiment with fresh CSS. We tested the hypothesis that freezing or ionising irradiation might have an impact on DNA extraction. E3 was the only method that gave a response so it was chosen for subsequent experiments.

Impact of physical treatments

Impact of freezing on bacterial DNA extraction

DNA extraction was performed on samples previously frozen at different steps and compared with a non-frozen sample (Fig. 1). Amplified products of each sample are shown in Fig. 5. Extraction of DNA from fresh CSS without any freezing step gave the best *rpoB* amplification results. Freezing CSS led to a very low recovery of bacterial DNA. Freezing the stomached suspension also reduced the concentration of PCR product but to a lesser extent than freezing the raw material.

To attempt to improve the reaction, a PCR was performed with bovine serum albumin (BSA) (Sigma, France) (1 mg ml^{-1}) which is known to neutralise inhibition compounds (Kreader, 1996). However, PCR amplification products obtained with or without BSA were completely identical (data not shown).

Results obtained from filtered or non-filtered mother suspensions demonstrated the need to filter suspensions.

Fig. 5 Impact of freezing, filtration and volume sample on bacterial DNA extraction on cold-smoked salmon. M: 5 µl of Mass Ruler^{TM} DNA ladder low range, MBI Fermentas, Lithuania; T-: negative control; *Cp*: positive control, DNA of the reference type strain of *Carnobacterium piscicola*

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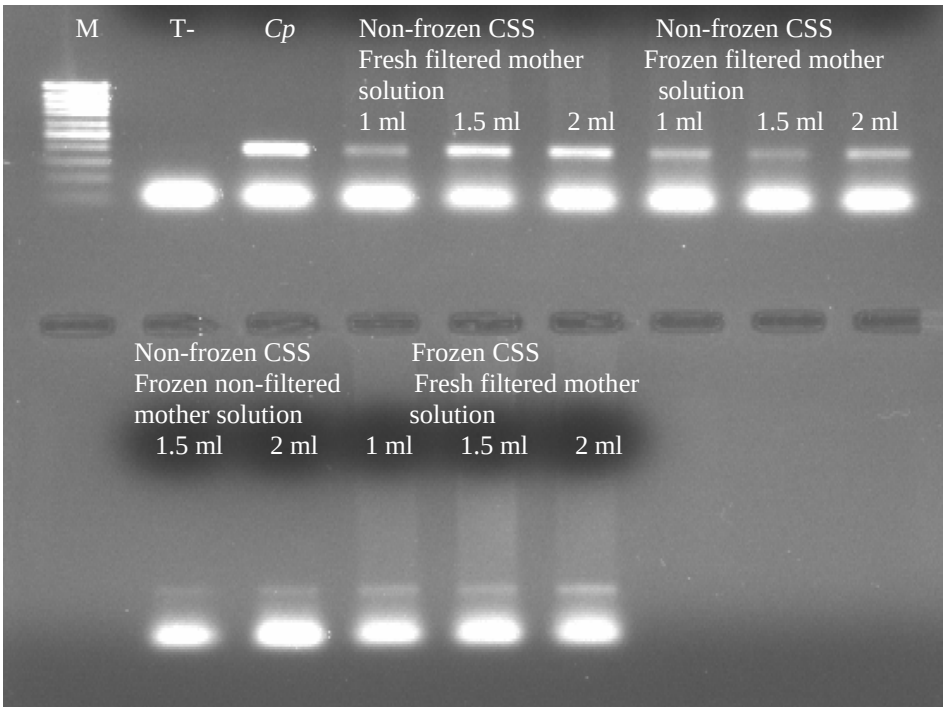

Impact of ionising irradiation on bacterial DNA extraction

The PCR reaction was performed on irradiated CSS samples and compared with non-irradiated samples (Fig. 2). PCR products from fresh non-treated CSS inoculated at 2.9 10^6 CFU g^{-1} gave a better signal (Fig. 6) than the other samples that were weakly positive (lanes 2 and 4) or negative (lane 3).

Fig. 6 Impact of ionising irradiation on bacterial DNA extraction on fresh or frozen cold-smoked salmon. M: 5 µl of Mass Ruler^{TM} DNA ladder low range, MBI Fermentas, Lithuania; *Cp*: positive control, DNA of the reference type strain of *Carnobacterium piscicola;* T-: negative control; lane 1*:* fresh cold-smoked salmon; lane 2: fresh ionised cold-smoked salmon; lane 3: frozen cold-smoked salmon; lane 4: frozen ionised cold-smoked salmon.

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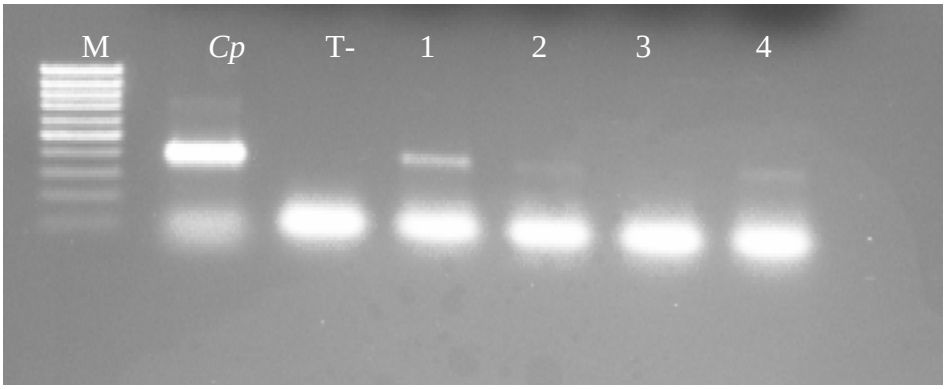

DISCUSSION

The cost of extraction based on the price of products and reagents was also taken into account and was significantly lower for E1 (1.7 euros) than for E2 and E3 (2.6 euros). However, in this estimation, personnel costs were not included. The lower price of the E1 extraction method is offset by a long procedure, particularly very long incubation times. E2 was less time-consuming than E1 but still much more than E3, which requires only 1 hour of incubation time. In addition, the E3 method can be used by inexperienced people unlike E1 and E2 which involve an unhealthy phenol/chloroform extraction step (see Table 1 for details).

Table 2: Analysis of the extraction costs (in euros) comparing the three methods.

E1						
		Quantity for a 100 ml stock solution	*Price/buying volume*	*Price in 100 ml stock solution*	*Price for 1 extraction (5 ml)*	*Total for 1 extraction*
Lysis solution	Tris-HCl	10 ml	24.08/1000 ml	0.24	0.012	*0.057*
	Na_2EDTA	1.86 g	106.09/1000 g	0.20	0.010	
	NaCl	0.58 g	55.64/1000 g	0.03	0.002	
	SDS	1 g	28.96/100 g	0.29	0.014	
	Sarkosyl	1 g	37.80/100 g	0.38	0.019	
Proteinase K		0.1 ml	172/20 ml at 20 g l^{-1}	0.1 ml		*0.86*
Phenol/ chloroform/ acid		5 ml	160/1000 ml	5 ml		*0.8*
						1.717
E2						
		Quantity for a 100 ml stock solution	*Price/buying volume*	*Price in 100 ml stock solution*	*Price for 1 extraction (0.5 ml)*	*Total for 1 extraction*
Stock solution (100 ml)	Na_2EDTA	3.72 g	106.09/1000 g	0.39	0.002	*0.072*
	GES	59.1 g	23.47/100 g	13.88	0.069	
	Sarkosyl	0.5 g	37.80/100 g	0.19	0.001	
		Quantity used for one extraction				
	lysozyme	25 mg	21/1 g			*0.53*
	mutanolysine	200 U	41.0/5000 units			*1.6404*
	ammonium acetate	250 µl	11.73/100 g		0.58/ ml	*0.15*
Stock solution 24:1 (25 ml)	Chloroform/ 2 pentanol		11.28/25 ml / 204.3/500 ml	10.83/ 0.41	0.22	*0.22*
						2.607
E3						
		Quantity for a 10 ml stock solution	*Price/buying volume*	*Price in 10 ml stock solution*	*Price for 1 extraction (180 µl)*	*Total for 1 extraction*
Lysis solution (10 ml)	Tris-HCl	1 ml	24.08/1000 ml	0.024	0.0004	*0.077*
	Na_2EDTA	0.186 g	106.09/1000 g	0.02	0.0004	
	lysozyme 20 mg ml^{-1}	200 mg	21/1 g	4.20	0.076	
	triton X 100	0.1 ml	21.49/100 ml	0.0215	0.0004	
Kit column						*2.50*
						2.577

Our results demonstrate that E3 is the most efficient method to detect bacterial DNA from CSS using a PCR-based reaction. Other studies have shown that DNA extraction kits give good results (McOrist et al., 2002; Li et al., 2003). McOrist and co-workers found that the use of the Qiamp DNA Stool Mini Kit resulted in a superior downstream performance in PCR compared with other methods applied in the same conditions. Studying factors affecting PCR detection, Aznar and Alarcón (2003) showed that the use of the DNeasy tissue kit (Qiagen) for DNA extraction improved PCR sensitivity and prevented the amplification of non-specific PCR products.
Concerning the impact of freezing, a preliminary study demonstrated, without explanation, that the freezing step could reduce the DNA content of thymus extract (Hajduk, 1999). As previously mentioned, there is still a lack of information concerning DNA changes in frozen food. Moreover, there are no studies focusing on bacterial DNA extraction and the impact of this physical treatment. Two hypotheses could be drawn; first, that compounds such as lipids or proteins might also be denatured or activated (nucleases) during the freezing period and interact in degrading bacterial DNA after the bacterial lysis step; second, that an inhibiting compound could be produced during the freezing period and extracted with bacterial DNA, leading to inhibition of Taq Polymerase. In a food matrix, many components are known to inhibit the PCR reaction, such as fish oil homogenate or NaCl (Rossen et al., 1992). To investigate this hypothesis, a PCR was performed with BSA but the reaction was not improved. Based on this result, we assume that the negative impact of freezing on bacterial DNA retrieval is probably not due to a PCR inhibition compound generated by freezing. The experiments related to ionising irradiation confirmed previous results regarding the impact of freezing, but also showed that ionising irradiation has a high negative impact on DNA extraction. To our knowledge, the impact of ionising irradiation on bacterial DNA extraction has never been reported.

In this experiment, we demonstrated that E3 was the most efficient of the three tested methods to extract bacterial DNA from CSS. A few modifications, such as using a filtration step and changing the reaction volume, improved the method. It appeared that the filtration step allowed the best recovery of bacterial DNA, probably by removing residual proteins and flesh. The best working volume was 2 ml of stomached suspension.
In those conditions, a good signal was obtained after *rpoB* PCR amplification.
Our experiments clearly show that physical treatments of CSS such as freezing and ionising irradiation have a negative impact on bacterial DNA recovery. An understanding of the molecular phenomena leading to these negative impacts has to be undertaken.
Considering these results, attention must be paid to food safety control using molecular analysis of bacteria when CSS or other food products are frozen before being commercialised. Freezing treatments are commonly used in food processing and irradiation occasionally. Thus bacterial detection by the PCR method could lead to a false negative response and hence a problem for the hygienic quality of food. In particular, this problem could be encountered in the detection of bacteria or viruses on biological samples without a pre-enrichment step.

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