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(54) Title: METHOD FOR DETECTING LIVE MICROBIOLOGICAL CONTAMINANTS IN A FOOD PRODUCT SAMPLE (54) Titre: PROCEDE DE MISE EN EVIDENCE DE CONTAMINANTS MICROBIOLOGIQUES VIVANTS DANS UN ECHANTILLON DE PRODUIT A USAGE ALIMENTAIRE (57) Abstract The invention concerns a method for detecting live microbiological contaminants in a food product sample which consists in detecting the presence of mRNA coding for the synthesis of a protein intervening in the synthesis of proteins by said contaminants, more particularly, mRNA coding for an elongation factor. (57) Abrégé La présente invention concerne un procédé de mise en évidence de contaminants microbiologiques vivants dans un échantillon de produit à usage alimentaire selon lequel on détecte dans ledit échantillon la présence d'ARNm codant pour la synthèse d'une protéine intervenant dans la synthèse des protéines par lesdits contaminants, plus particulièrement, d'ARNm codant pour un facteur d'élongation.		

PROCESS FOR DETECTING LIVE MICROBIOLOGICAL CONTAMINANTS
IN A FOOD PRODUCT SAMPLE

The present invention relates to a process for
5 detecting live microbiological contaminants in a food
product sample.

Food products are particularly susceptible to
contamination with microbiological products, in
particular with bacteria, on account of the powerful
10 effect which a contamination has on the health of the
persons ingesting the food. In this sphere, live, and
therefore active, microorganisms are particularly
formidable since they are then able to propagate in the
body and transmit severe diseases. There is therefore a
15 definite need to develop a process which enables these
live microorganisms to be detected in food products in
a manner which is both precise and satisfactory.

A large number of methods exist for detecting
bacteria, in particular, in samples. One which may be
20 mentioned, by way of example, is that of culturing the
sample in order to increase the number of bacterial
cells present until colonies, which can be counted, are
observed. Where appropriate, the culture can be
followed by an additional step which enables the
25 particular type of bacteria contained in the sample to
be identified. These bacteriological methods require a
great deal of time and skill on the part of the
individuals who carry them out. Thus, it is generally
necessary, for example, to incubate for from 24 to
30 48 hours before being certain of obtaining a positive
or negative result.

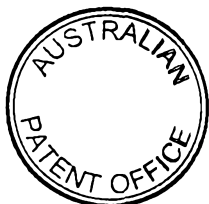
Other methods have also been envisaged for
eliminating the drawbacks of the conventional
bacteriological method. Thus, microscopy is frequently
35 used for detecting bacteria in clinical samples.
Usually, it is necessary to stain the sample in order
to increase the detection limits, a procedure which on
the one hand represents a laborious method and, on the
other hand, is unsuitable for food samples (1). Some



immunological methods have also been successfully developed for detecting certain species which possess surface antigens which can be recognized by specific antibodies. However, such an approach cannot be used
5 for qualitatively determining bacteria in a sample which may contain a large variety of bacterial species which do not possess a common surface antigen.

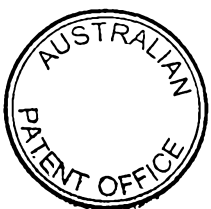
The European Patent Application EP-A-0 133671 describes a method for determining the presence of
10 bacteria in samples, in particular in media, such as body fluids, which are suitable for diagnostic purposes, which employs nucleic acid hybridization techniques. According to this method, the sample to be tested is first of all subjected to denaturing
15 conditions so as to render the nucleic acids of the bacteria, which are present in the sample, single-stranded. The resulting single-stranded nucleic acids are brought into contact with a polynucleotide probe which possesses a sequence which is homologous with at
20 least one sequence which is common to all the bacterial species which are present in the sample. The probe and the denatured nucleic acids are brought into contact so as to effect a hybridization between the probe and the respective sequences of the bacteria. More
25 particularly, the probe employed comprises at least a part of one of the strands of a gene which codes for the synthesis of a nucleic acid or a protein which is involved in the mechanism by which the proteins are synthesized. Those genes of this type which are cited
30 are, in particular, genes which encode transfer RNAs, ribosomal RNAs, or initiation, elongation or translation termination factors. One of the features of this method is

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35 that it is not based on expression but rather on the presence of hybridizable nucleic acids, for example the RNA or the genomic DNA or the extra-chromosomal nucleic acids of the bacteria which are present. The result, which the Applicant views as being an advantage, of this is that the samples do not have



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to be treated so as to guarantee the viability of the bacteria which are present.

Document WO 92/03455 describes compositions and processes for treating and diagnosing infections with
5 Candida, in particular nucleotides which are able to hybridize specifically with a part of the Candida mRNA, in particular the mRNA encoding elongation factors 1 and 2 (TEF1 and TEF2). This document is only directed towards therapeutic or diagnostic applications, either
10 for detecting the presence of Candida in a patient or for inhibiting the activity of this bacterium by blocking the expression of essential proteins.

The document Berg et al., "MOL CELL. PROBES, Vol. 10, February 1996 , pp 7-14" describes a system
15 for specifically detecting the DNA of microplasmas using the PCR technique. Although it is indicated, on page 12 of this document, that the target sequence for the PCR amplification is the tuf gene, which encodes the elongation factor Tu, this document is only
20 directed towards detecting DNA, and not mRNA on the one hand, and, on the other hand, the method is only a method for detecting bacteria in order to establish a diagnosis in a patient. This document does not envisage any application in the sphere of the invention, in
25 which the specific problem is that of detecting living bacteria.

Thus, there is no known method which makes it possible to detect live microbiological contaminants in a food product and which at the same time discriminates
30 between the live microorganisms and the dead microorganisms and which, moreover, does not pose any problems relating to public health.

The inventors have now discovered that it was possible to detect live microbiological contaminants in
35 a food product sample by detecting, in this sample, the presence of messenger RNA (mRNA) which codes for the synthesis of a protein which is involved in the



mechanism by which the proteins of the said contaminants are synthesized.

Different families of microorganisms can be detected in accordance with the invention. Procaryotes, in particular bacteria, unicellular eucaryotes, in particular yeasts, and multicellular eucaryotes, in particular fungi, may be mentioned in a nonlimiting manner. Different species can be identified within these families. Thus, for example, Escherichia, Salmonella and Mycobacterium in the case of bacteria; Saccharomyces and Candida in the case of yeasts; Mucor, Neurospora and Trichoderma in the case of fungi.

The synthesis of proteins by microorganisms comprises steps of transcription and translation. Within the context of translation, nucleic acids and proteins exist which are involved in each of the three basic steps of protein synthesis, i.e. initiation, elongation and termination.

It is thus possible to mention, as elements involved in this stage of translation, transfer RNAs, ribosomal RNAs, ribosomal proteins, and initiation, elongation and termination factors. In fact, proteins or nucleic acids exist whose primary structure is at least partially conserved between different microorganism species belonging to one and the same family, and this property is advantageously exploited in accordance with the



invention in order to detect live microbiological contaminants belonging to different species.

Thus, according to one preferred embodiment, the invention relates to a process for non-specifically
5 detecting live contaminants belonging to different species of a family of microorganisms, according to which the mRNA detected is an mRNA which codes for the synthesis of a protein whose primary structure is at least partially conserved between different species.

10 On the contrary, if a greater specificity of detection between different microorganism species is required, it is possible, according to the invention, to detect different mRNAs which respectively code for the synthesis of a protein whose primary structure is
15 not conserved between different species.

Of all the factors which are involved in protein synthesis, one example which is particularly preferred consists of the elongation factors. Those of these factors which may be mentioned are the EF-1,
20 EF-2, EF-G and EF-TU factors, in the case of bacteria (2), or else the EF-1 α factor (3) in the case of yeasts and fungi. These factors play a fundamental role in protein synthesis in that they determine the length of time during which an aminoacyl tRNA remains associated
25 with the ribosome and with the forming polypeptide chain, which function is referred to by the expression "kinetic proofreading" (4).

There are various reasons why it is particularly advantageous to look for the presence of
30 messenger RNA which encodes an elongation factor. First of all, this gene represents a very suitable marker of the viability of the cells since inactivation of this gene is a lethal event both in procaryotes and eucaryotes (5, 6). Furthermore, the gene which encodes
35 an elongation factor encodes a protein which belongs to those proteins which are most widely expressed in procaryotes and eucaryotes (7, 8), a fact which makes it possible to substantially decrease the cell detection level. Finally, as pointed out above, it is



possible to modulate the specificity of the detection insofar as this function is conserved in procaryotes and eucaryotes and the primary structure of this type of gene is very similar (9). It is thus possible to
5 implement means of detection which make it possible to distinguish between procaryotes and eucaryotes or, on the contrary, to non-specifically detect bacteria, yeasts and/or fungi at one and the same time.

Furthermore, mRNA encoding elongation factors
10 has a very short half-life (10, 11). Its presence therefore reveals the presence of cells which were still alive approximately ten minutes before the mRNA was detected.

An mRNA encoding an elongation factor is
15 therefore detected in accordance with a preferred embodiment. In this case, a live microorganism cell within the meaning of the invention is a cell which is able to produce the mRNA corresponding to an elongation factor.

20 The RT-PCR (polymerase chain reaction combined with reverse transcription) is a method of choice for detecting the presence of messenger RNA according to the invention. This technique consists in carrying out a PCR on an RNA which has previously been transcribed
25 into complementary DNA in the presence of reverse transcriptase and a primer. After the RT stage, the proper PCR stage is carried out under standard conditions in the presence of the DNA to be amplified, two oligonucleotide primers which flank the region to
30 be amplified and four deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), in large molar excess, and the enzyme Taq polymerase.

Naturally, the choice of the primers is a basic requirement, since it makes it possible to target the
35 mRNA which it is desired to detect. The oligonucleotide primers are prepared such that they are specific for the coding region of a gene which encodes an elongation factor. The known elongation factors which are preferably selected are the EF-TU factor in the case of



bacteria and the EF-1 α factor in the case of yeasts and fungi. It is for this reason that the primers B1/B2 (5' CGCTGGAAGGCGACGMRRAG 3'/5' CGGAAGTAGAACTGCGGACGGTAG 3') were, for example, prepared, which primers are specific for a fragment of the bacterial EF-TU elongation factor which is found, in particular, in *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Escherichia coli*, *Brevibacterium linens* and *Streptomyces ramocissimus*.

In the case of yeasts, the primers L1/L2 (5' TCCATGGTACAAGGGTTGGGAA 3'/5' GCGAATCTACCTAATGGTGGGT 3') were prepared, which primers are specific for a fragment of the yeast EF-1 α elongation factor, which is found both in *Saccharomyces cerevisiae* and *Candida albicans*.

Finally, an example of a nucleotide primer which can be used for detecting messenger RNA which is specific for fungi consists of the pair M1/M2 (5' GCTGGTATCTCCAAGGATGG 3'/5' - CGACGGACTTGACTTCRGTGG 3').

These primers are more particularly specific for a fragment of the fungal EF-1 α elongation factor which is found in *Mucor racemosus*, *Neurospora crassa*, *Trichoderma reesei*, *Absidia glauca*, *Aureobasidium pullulans*, *Histoplasma capsulatum* and *Puccinia graminis*.

The primers which can be used in accordance with the invention were prepared after comparing the elongation factor-encoding regions of different microorganism species (Lasergene software, Dnastar, Madison, Wisconsin, USA).

In a general manner, the process according to the invention can be characterized by the following steps taken together:

- a) a sample of the food product to be tested is withdrawn;
- b) the cells are lysed;
- c) reverse transcription is carried out;
- d) amplification cycles are carried out using oligonucleotide primers which are specific for



the coding region of a gene which encodes an elongation factor;

e) the amplification products are separated;

f) the amplification products are visualized.

5 g) the products as visualized in step f) are compared with the amplification products which are obtained from pure mRNA.

Since the invention is essentially directed towards detecting live microbiological contaminants, it is particularly advantageous to as far as possible remove any additional contamination of the medium with the DNA which is present in the sample. For this reason, according to a preferred embodiment of the invention, an additional step (b'), which is intended to remove the DNA which is present in the sample, is added after step b). For this, DNase I, which does not contain any RNase, can simply be added to the reaction medium. If it is desired to check for the absence of false positives which are linked to the presence of DNA, one possibility consists in carrying out a PCR reaction on the same samples as those used for the RT-PCR.

The invention will be illustrated in more detail by the embodiments which follow and which are accompanied by Figures 1 to 3.

Figure 1 depicts an electrophoresis gel of the RT-PCR products of the EF-Tu gene from *E. coli* (tuf b gene) (A) or of the EF-1 α gene from *S. cerevisiae* (B) or from *M. racemosus* (C), suspended in milk in concentrations of from 10⁵ cells/ml (lane 1) to 1 cell/ml (lane 6), while decreasing the concentration 10 times on each occasion. Lane 7 depicts a negative control without cells; lane 8 depicts a positive control with pure mRNA; lane 9 depicts a PCR which was carried out on a lysate which was digested with DNase I; the M lanes depict a 1kb ladder (Gibco BRLTM).

Figure 2 depicts an electrophoresis gel of the RT-PCR products of the EF-Tu gene from *E. coli* (tuf b gene) (A) or of the EF-1 α gene from *S. cerevisiae* (B)



or from *M. racemosus* (C), suspended in milk at a cell concentration of 10⁴ -10⁵ cells/ml. Lane 1 corresponds to 100% dead cells; lane 2 to 25% live cells and 75% dead cells; lane 3 to 50% dead cells and 50% live cells; lane 4 to 75% live cells and 25% dead cells; lane 5 to 100% live cells; lane 6 to a negative control without cells; lane 7 to a positive control with pure mRNA; lane 8 to a PCR carried out on a lysate digested with DNase (I); and lanes M to a 1kb ladder (Gibco BRLTM).

Figure 3 depicts an electrophoresis gel of the RT-PCR products of the EF-Tu gene from *E. coli* (*tuf b* gene) and of the EF-1 α gene from *S. cerevisiae* and *M. racemosus*. The gel is obtained in one single RT-PCR step. The cells were suspended in milk at a concentration of 10 cells/ml. Lane 1, bacterial primers; lane 2, yeast primers; lane 3, fungal primers; lane 4, bacterial and yeast primers; lane 5, yeast and fungal primers; lane 6, bacterial and fungal primers; lane 7, bacterial, yeast and fungal primers; lane 8, negative control without cells; lane 9, positive control with pure mRNA; lane 10, PCR carried out on a lysate digested with DNase (I); M lanes, 1kb ladder (Gibco BRLTM).

Examples were carried out with the following strains:

- *Escherichia coli* (strain ATCC 19110);
- *Saccharomyces cerevisiae* S288C;
- *Mucor racemosus* (strain CBS 11308).

While the sample on which the process was performed is milk, the invention can of course be applied to any other, preferably liquid, food product.

EXAMPLE 1 : determining the presence of live *E. coli* cells in milk (detecting the mRNA encoding the EF-Tu elongation factor)

a) Preparing the strain

An *E. coli* strain is cultured at 37°C in Luria broth, and then a solid medium is prepared by adding 2% agar (Difco Laboratories). A portion of the cells is



removed during the exponential phase of growth. The cells are divided up into samples of 2 ml and then subjected to lethal conditions by incubating them at 65°C for 15 min. Dilution series of the samples are
5 plated out on agar medium in order to check the viability of the cells and to count them. Finally, the samples are used for inoculating samples of commercially available cow's milk which has been pasteurized at ultrahigh temperature.

10 b) Contaminating the milk

Concentration series of from 105 to one cell per ml of milk are made up. The possible interference of the milk in the PCR reaction is ruled out by carrying out 4 cycles of washing with PBS medium, as
15 indicated in the Cooray et al. document (12), the contents of which is hereby incorporated by reference. After washing, the final milk pellet is resuspended in a volume of PBS equal to that of the original volume of milk, and this suspension is then filtered through a
20 25 mm-diameter polycarbonate membrane which has been moistened with PBS medium (0.8 µm pore size, Millipore). The filter is transferred into a 15 ml Falcon™ tube (Becton Dickinson). 1 ml of PBS is added and the whole is mixed vigorously for 30 seconds, after
25 which the supernatant is recovered. A further 0.5 ml of PBS is then added to the filter and the resulting suspensions are combined together and then centrifuged at 5000 g for 5 min.

40 units of RNase inhibitor (Boehringer) are
30 added to prevent the RNA being degraded, and water, which has been adjusted with 0.05% diethyl pyrocarbonate in order to inactivate nucleases, is added to a final volume of 50 µl, in accordance with the method described in Roszak et al. (13), the content
35 of which is hereby incorporated by reference.

The cells are then lysed by adding 50 mg of glass microbeads (0.5 mm, Biospec Products, Bartlesville, Oklahoma). The tubes are shaken twice, with a 10 second interval, at 5000 rpm for 20 seconds,



and are left on ice between two mixings. After decantation, 10 µl of the lysate is transferred to a new tube.

c) Removing the genomic DNA

5 EZ buffer (Perkin Elmer kit), as reaction buffer for the 1-times concentrated rTth enzyme, 2.5 mmol of Mn(OAc)₂ and, finally, 10 units of RNase-free DNase (I) (Boehringer), in order to prevent subsequent amplification of contaminating genomic sequences (14), are added to give a final volume of 10 20 µl. After incubating at 37°C for 10 min, the DNase is inactivated by raising the temperature to 95°C for 5 min.

d) RT-PCR

15 The two steps of RT and of PCR are carried out consecutively in the same tube. For the RT step, the following are added in order to give a final volume of 50 µl: 15 µl of lysate digested with DNase I, 1 x EZ buffer, 1.4 mM of Mn(OAc)₂, 0.3 mM of 20 deoxyribonucleoside triphosphate (Perkin Elmer), 0.4 µM of primers B1 and B2 (5' CGCTGGAAGGCGACGMRRAG 3'/5' CGGAAGTAGAACTGCGGACGGTAG 3') (upper and lower strands) and 5 units of rTth DNA polymerase (Perkin Elmer). The mixture is incubated for 2.5 min at 95°C, 20 min at 25 60°C and 1 min at 95°C. For the PCR step, 40 cycles are carried out directly, with each cycle consisting of 15 seconds at 95°C and 30 seconds at 60°C. A final extension is carried out for 10 min at 60°C.

e) Detecting false positives

30 Checking to determine whether there are any false positives due to contamination with DNA is carried out by adding 5 µl of the DNase I-digested lysate, 1 x Taq buffer (Boehringer), 0.05 mM of deoxyribonucleoside triphosphate, 0.4 µM of each of the 35 primers B1 and B2, and 2.5U of Taq polymerase (Boehringer) to give a final volume of 50 µl.

f) Separating the amplification products

10 µl of the amplification products are separated by horizontal gel electrophoresis (1 to 2%



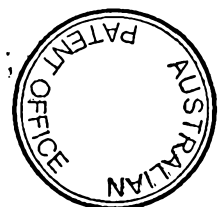
agarose in EDTA tris-acetate), and the bands are visualized by staining with ethidium bromide and illuminating with UV light. Total RNA is also analyzed as a positive RT-PCR control (it is extracted using the
5 RNAgent™ kit (Promega)), and the resulting amplification products are compared with those obtained from the total RNA. An optional step can be added in order to confirm the identity of the amplified products. For this, the bands of expected size are
10 purified on a gel ("Gel Extract Kit", Qiagen) (462 bp fragment), and 1 µl of the DNA solution is then used as the template for a second PCR employing internal primers.

Results

15 It was possible to detect the elongation factor using 10 cells/ml, which corresponds to 5-10 cells per reaction tube in accordance with the procedure employed (Fig. 1A, lane 5). No transcript is detected below this level or in uncontaminated milk (Fig. 1A, lanes 6 and
20 7, respectively). No detectable PCR product appeared when using the cell lysate following treatment with the DNase I (Fig. 1A, lane 9). It therefore appears that adding a step of treatment with DNase I does indeed prevent contaminating genomic sequences from being
25 amplified.

EXAMPLE 2 : Carrying out an RT-PCR on a milk sample which is contaminated both with living and dead E. coli cells

When all the cells are dead (this was checked
30 by plating out), the RT-PCR detection is negative (Fig. 2A, lane 1), which confirms that the RT-PCR method makes it possible to only detect living cells. Although producing quantitative results is difficult using conventional RT-PCR, gel electrophoresis analysis
35 demonstrated a quantitative increase in the intensity of the specific bands when the number of viable cells increases (Fig. 2A, lanes 2 to 5), indicating an apparent increase in the target DNA of the RT-PCR procedure as the number of viable cells increases.



EXAMPLE 3 : Determining the presence of living *S. cerevisiae* cells in milk (detecting the mRNA encoding the EF-1 α elongation factor).

The procedure of Example 1 is repeated apart
5 from the following differences:

An *S. cerevisiae* S 288C strain is cultured at 30°C in a YPD ("yeast peptone dextrose") medium containing 2% bactopectone, 1% yeast extract (Difco Laboratories) and 2% glucose. A solid medium is then
10 prepared by adding 2% agar (Difco Laboratories). A portion of the cells is withdrawn during the exponential phase of growth. The cells are divided up into samples of 2 ml and then exposed to lethal conditions by being incubated at 65°C for 15 min.

15 When the RT-PCR is carried out, the following EF-1 α elongation factor-specific oligonucleotide primers are employed:

L1 : 5' TCCATGGTACAAGGGTTGGGAA 3'

L2 : 5' GCGAATCTACCTAATGGTGGGT 3'

20 The same type of result is obtained as in Example 1 (Fig. 1B, detecting a fragment of 657 bp in size).

EXAMPLE 4 : Carrying out the RT-PCR on a milk sample which is contaminated with both living and dead
25 *S. cerevisiae* cells.

The same type of results is observed as in Example 2 (Fig. 2B).

EXAMPLE 5 : Determining the presence of living
30 *M. racemosus* cells in milk (detecting the mRNA encoding the EF-1 α elongation factor).

The procedure of Example 1 is repeated except that, in step a), the *M. racemosus* strain is cultured at 30°C in a YPD medium containing 2% bactopectone, 1% yeast extract (Difco Laboratories) and 2% glucose. A
35 solid medium is then prepared by adding 2% agar (Difco Laboratories). A portion of the cells is withdrawn during the exponential phase of growth. The cells are divided up into samples of 2 ml and then exposed to



lethal conditions by incubating them at 120°C for 30 min.

Serial dilutions of the samples are plated out on OGY agar medium (MERCK) in order to check the viability of the cells and to count them.

When the RT-PCR is being carried out, the following EF-1 α elongation factor-specific nucleotide primers are employed:

M1 : 5' GCTGGTATCTCCAAGGATGG 3'

M2 : 5' -CGACGGACTTGACTTCRGTGG 3'

The same type of result is obtained as in Example 1 (Fig. 1C, determining a fragment of 498 bp in size).

EXAMPLE 6 : Carrying out the RT-PCR on a sample of milk which is contaminated with both living and dead *M. racemosus* cells.

The same type of result is observed as in Example 2 (Fig. 2C).

EXAMPLE 7 : Determining the simultaneous presence of living *E. coli*, *S. cerevisiae* and *M. racemosus* cells.

The EF-Tu gene of *E. coli* and the EF-1 α genes of *S. cerevisiae* and *M. racemosus* were detected simultaneously in one single reaction. The amplification products of expected size (fragments of 462 bp (*E. coli*) 675 bp (*S. cerevisiae*) and 498 bp (*M. racemosus*) in size, respectively) were detected simultaneously by means of RT-PCR using milk which was contaminated at one and same time with *E. coli*, *S. cerevisiae* and *M. racemosus*. For this, two (Fig. 3, lanes 4, 5 and 6) or three (Fig. 3, lane 7) pairs of primers were added to the same reaction mixture without modifying the RT-PCR conditions.

Thus, it appears that RT-PCR is a particularly powerful method for detecting viable contaminants on account of its great ability to increase detection sensitivities and the speed at which it can be carried out. It was possible to achieve a level of detection of 10 cells/ml of milk, and the whole of the RT-PCR process, including washing and filtering the milk



sample, was performed in 4 hours without there being a need for a preliminary enrichment step. The choice of the target gene is important. It has thus been found to be particularly advantageous to select primers which are specific for the coding region of the elongation factor gene.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (A) NAME: COMPAGNIE GERVAIS DANONE
(B) STREET: 126/130 RUE JULES GUESDE
(C) CITY: LEVALLOIS PERRET
(E) COUNTRY: FRANCE
(F) POSTCODE: 92300

10

(ii) TITLE OF THE INVENTION: PROCESS FOR DETECTING
LIVE MICROBIOLOGICAL CONTAMINANTS IN A FOOD
PRODUCT SAMPLE

15 (iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version
#1.30 (OEB)

(2) INFORMATION FOR SEQ ID NO: 1:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

35

CGCTGGAAGG CGACGMRRAG

20

(2) INFORMATION FOR SEQ ID NO: 2:



(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

10

CGGAAGTAGA ACTGCGGACG GTAG

24

(2) INFORMATION FOR SEQ ID NO: 3:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

25 TCCATGGTAC AAGGGTTGGG AA

22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 22 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGAATCTAC CTAATGGTGG GT

22



(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

15 GCTGGTATCT CCAAGGATGG 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGACGGACTT GACTTCRGTG G 21



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

- 5 1. Process for detecting live microbiological contaminants in a food product sample, according to which the presence of mRNA encoding a protein involved in the synthesis of proteins produced by said contaminants is detected in said sample.
2. Process according to claim 1 for non-specifically detecting live contaminants
10 belonging to different species of a family of microorganisms, wherein said mRNA codes for the synthesis of a protein whose primary structure is at least partially conserved between the said different species.
3. Process according to claim 1 for specifically detecting live contaminants
15 belonging to different species of a family of microorganisms, wherein the presence is detected of different mRNAs, each of which codes for the synthesis of a protein whose primary structure is not conserved between the said different species.
4. Process according to any one of claims 1 to 3, wherein said mRNA encodes
20 an elongation factor.
5. Process according to any one of claims 1 to 4, wherein the presence of mRNA is detected by performing the RT-PCR technique.
- 25 6. Process according to claim 5, wherein the oligonucleotide primers employed in the PCR are specific for the coding region of a gene which encodes an elongation factor.
7. Process according to claim 6 for detecting bacteria, wherein the said
30 elongation factor is the EF-Tu factor.



8. Process according to claim 7, wherein the following pair of oligonucleotide primers is employed:

5

B1 : 5' CGCTGGAAGGCGACGMRRAG 3'
B2 : 5' CGGAAGTAGAACTGCGGACGGTAG 3')

9. Process according to claim 6 for detecting yeasts or fungi, wherein the said
10 elongation factor is the factor EF-1 α .

10. Process according to claim 9 for detecting yeasts, wherein the following pair of oligonucleotide primers is employed:

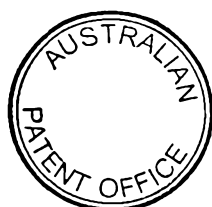
15 L1 : 5' TCCATGGTACAAGGGTTGGGAA 3'
L2 : 5' GCGAATCTACCTAATGGTGGGT 3'

11. Process according to claim 9 for detecting fungi, wherein the following pair of oligonucleotide primers is employed:

20 M1 : 5' GCTGGTATCTCCAAGGATGG 3'
M2 : 5' -CGACGGACTTGACTTCRGTGG 3'

12. Process according to any one of claims 1 to 11, wherein:

- 25 a) a sample of the food product to be tested is withdrawn;
b) the cells are lysed;
c) a reverse transcription is carried out;
d) amplification cycles are carried out using oligonucleotide primers which are specific for the coding region of a gene which encodes an elongation factor;
30 e) the amplification products are separated;
f) the amplification products are visualized;



g) the products as visualized in step f) are compared with the amplification products which are obtained from pure mRNA.

5 13. Process according to claim 12, wherein a step b'), which is directed toward removing the DNA which is present in the sample, is added after step b).

14. Process according to claim 13, wherein it includes a step which is directed toward verifying the absence of false positives linked to the presence of DNA.

10

15. A process according to any one of claims 5 to 14 for specifically detecting bacteria and/or yeasts and/or fungi, wherein at least two primer pairs selected from B1/B2, L1/L2 and M1/M2 as hereinbefore described are employed in the RT-PCR.

15 16. A process according to claim 1, substantially as hereinbefore described with reference to any one of the Examples.

DATED: 21 August 2000

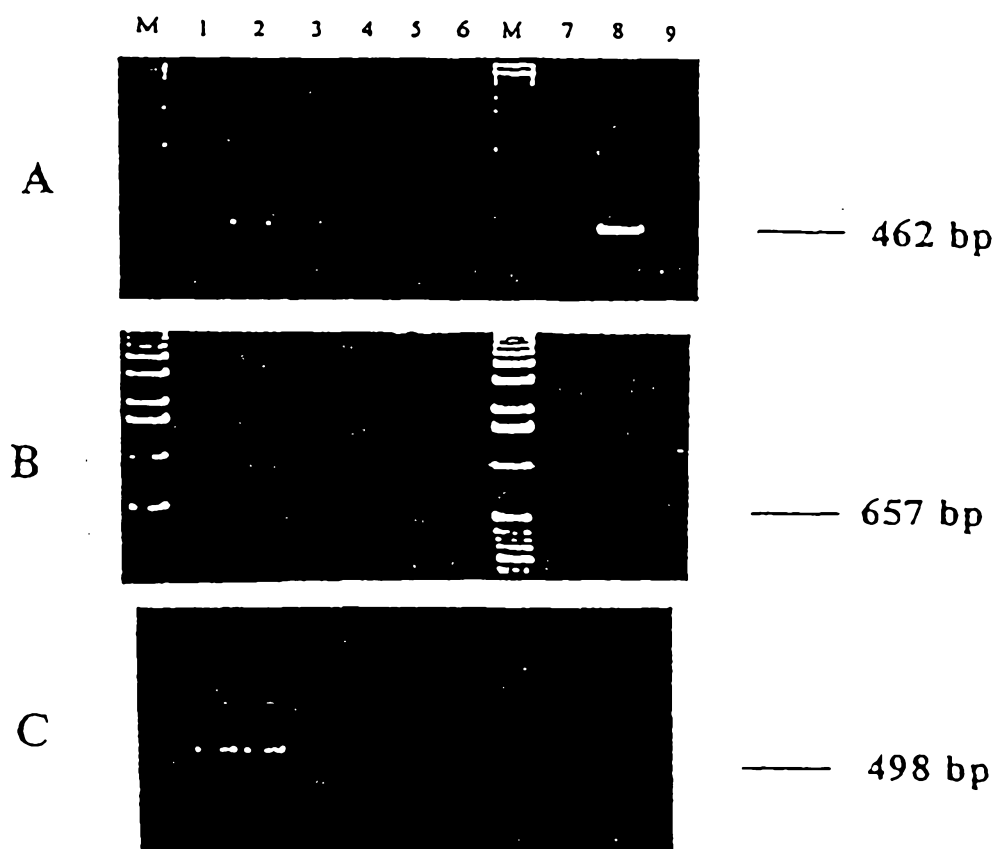
20 PHILLIPS ORMONDE & FITZPATRICK

Attorneys for:

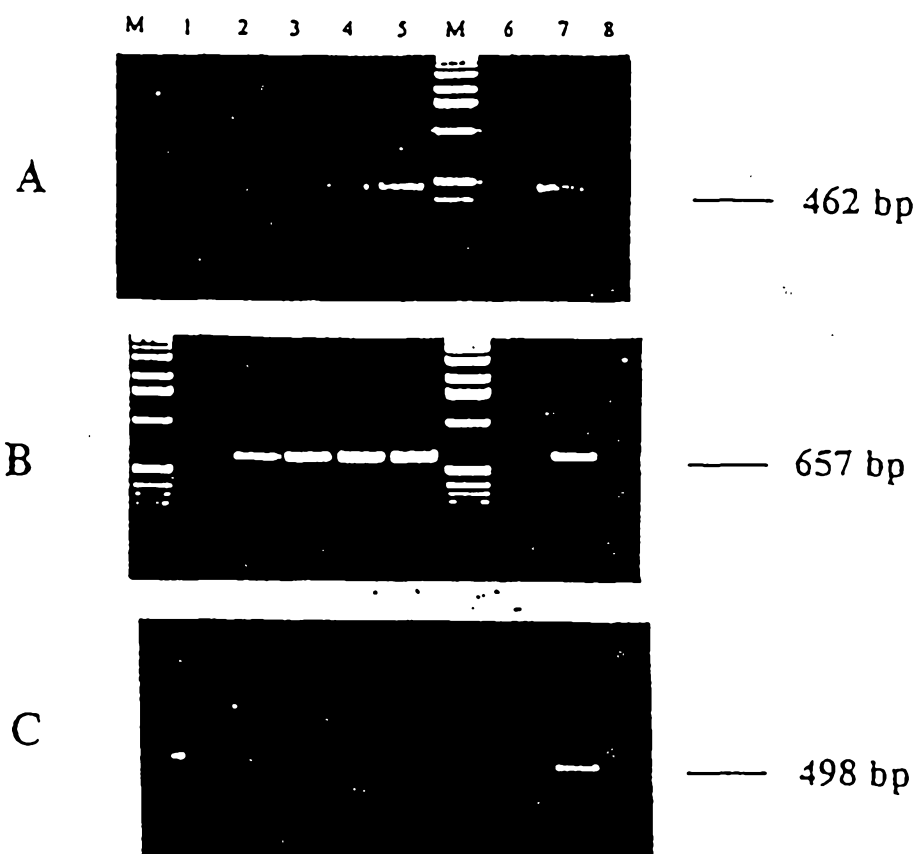
COMPAGNIE GERVAIS DANONE



1/ 3

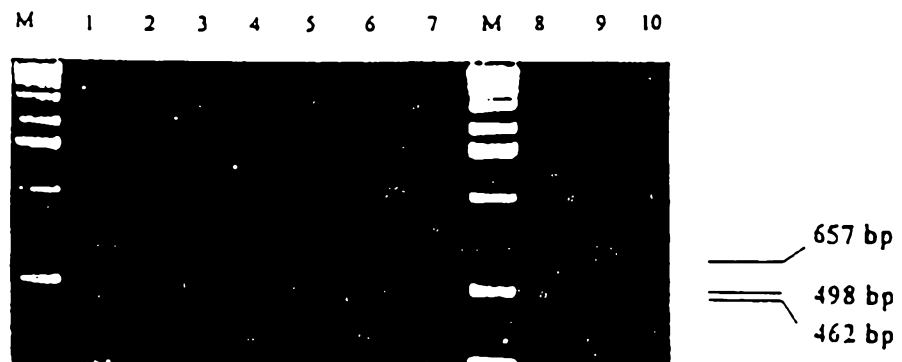
FIG_1

2 / 3



FIG_2

3 / 3

FIG_3