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An improved method for the detection of biogenic amines producing bacteria in fish

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ABSTRACT

This work concerns the development of rapid and reliable method for the isolation and characterization of bacteria producing biogenic amines for two types of fishes, the sardine (Sardina pilchardus) and the wolf fish (Dicentrarchus labrax). We proposed new an experimental protocol with two steps: The first step searching the genes responsible for the production of biogenic amines the genetic methods, including the ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction) and the Rep-PCR (Repetitive Extragenic Palindromic Polymerase Chain Reaction). The second step searching confirmation of the capacity of the genes identified to produce biogenic amines by chromatograph methods including the thin layer chromatography (TLC) for semi-quantitative test and high performance liquid chromatography (HPLC) for quantitative test. The results found showed that the sequencing of these steps allows the isolation and the rapid and accurate characterization of detecting the biogenic amine producing bacteria in fish. The comparison of the two kinds of fish used confirms that sardine (red fleshed fish) disseminates the histamine producing bacteria more than the wolf (white fleshed fish), explaining the high involvement of sardine in histamine intoxication.

Keywords: Biogenic amines, ERIC-PCR, Rep-PCR, HPLC, sardine, red flesh fish, fish wolf.

INTRODUCTION

The biogenic amines are well known for their implication in serious human intoxications, associated with the consumption of spoiled fish. Even though the isolation and characterization of bacterial strains producing biogenic amines in general and particularly histamine knew a great evolution, the methods used for their detection in foods depend on many parameters, such as the nature of food and the bacterial flora. The early methods used were based on the measure of the carbon dioxide produced with the decarboxylation of amino acids. This process is inaccurate and is now abandoned. Thereafter, several authors proposed the use of selective culture media, in order to obtain a rapid selection of the biogenic amines producing bacteria, such as trypticase soya broth enriched with 2% of histidine for the Enterobacteria [1], and the De Man Rogosa and Sharpe (MRS) medium used for the isolation of lactobacilli [2], which is adequate for the development of lactobacilli producing histamine and tyramine when it is enriched with histidine and tyrosine, respectively. Other studies are based on the criteria of the color change of the culture media after their inoculation [3, 4, 5]. However, these methods remain fastidious and inaccurate. For example, the same color produced in the culture medium may be relayed to various biogenic amines. The rise in the pH of the medium is not the exclusive result of the production of biogenic amines. Furthermore, the bacterial growth, in certain cases, could lead to a relatively low pH, without the production of enough biogenic amines to assure the color change in the medium. Hence, the development of new rapid and more accurate methods becomes necessary.

Currently the introduction of the genetic methods, such as Polymerase Chain Reaction (PCR), as a direct method of detection of biogenic amines producing bacteria, may lead to a great improvement of the experimental protocol, and to obtain more accurate and reliable results [6]. The presence of gene coding the amino acid decarboxylase does not

represent the production of the amine in the culture medium. So the use of thin layer chromatography (TLC) remains a semi-quantitative method used for the description of the produced amine but the highly liquid Chromatography performance (HPLC) remains a complementary method for confirmation, which indicates at the same time the presence and the rate of accumulation of biogenic amines in foods. The main objective of this work was to develop an improved method (the genetic methods) based on PCR for rapid and accurate results of detection of biogenic amines producing bacteria in fish for the fish industry. In this paper we proposed a new, easy and short protocol (two steps) for isolation and identification of genes responsible of the biogenic amine production.

MATERIALS AND METHODS

The protocol for isolation of the biogenic amines producing bacteria proposed three steps, requiring duration of at least three days, it is proposed by Afilal and Zlaiji but it giving no precise results [7]. In this work, we propose to modify this experimental protocol to produce rapid and more reliable results. After the isolation and the purification of the bacterial strains, we proposed the first step is the Multiplex-PCR for the identification of genes responsible of the biogenic amine production.

Isolation and purification of bacteria:
The isolation and purification of bacteria were performed on samples of fresh sardine and wolf, purchased in Oujda area and transported at 4 °C to the laboratory. For the isolation of bacteria, 3 subjects of sardine and one of the wolf were ground, separately, with ultra-turax during 2 min, and then 10g of each fish sample were introduced in 90 ml of sterile and neutral (pH: 7) physiological water. The solutions obtained were then used for the preparation of successive decimal dilutions in sterile physiological water. A volume of 0,1ml of each dilution was pouring plated on the culture medium corresponding to the microbial group. The medium Punt Count Agar (Merck) was used for the Standard Plate Count (SPC), the medium Mac Conkey (Biolife, Italy) was used for Enterobacteria and the medium of De Man Rogosa and Shape (MRS) for lactic bacteria [3]. The incubation was done at 30°C for 3 days. The bacterial strains isolated from each medium were stored at 4°C on slants of their corresponding medium in tubes, for further studies.

Identification of genes responsible on the biogenic amines production:
In this study, we used specific primers for the identification of the following genes: histidine decarboxylase (hdc-f, hdc-r) for Gram negative bacteria [8], histidine decarboxylase (HDC-3, HDC-4) for the Gram positive bacteria [6], ornithine decarboxylase (odc-3, odc-16) and tyrosine decarboxylase (P1-rev, P2-for) [9], coding for fragments of 709bp, 440bp, 1446bp and 924bp, respectively. For the amplification of DNA we used a colony direct multiplex PCR method as described by Coton and al in 2005[6]. The mixture of the amplification, of a volume of 25µl, contains a colony, 20 mM of Tris-HCl, pH 8.0, 50 mM of KCl, 2.5 mM of MgCl2, lM of each dNTP, and 1U of DNA polymerase (Ampli GoTaq). Each primer was used in the proportion of 1µM [8]. The parameters of amplification used are the same as described by Marcobal et al. in 2003 and Blanca and al. in 2005 [9, 10].

Detection of biogenic amines with chromatograph methods (TLC and HPLC)
For the separation of the biogenic amines with TLC, the method used was described by lot of authors [7, 11]. The solvent system used was the Benzene Triethylamine (5: 1) [12] and the revelation was done by UV with a wavelength of 254 nm. The stationary phase was silica gel on ready to use plates (20X20 cm) (Poly Gram Sil G, Merck) with thickness of 0.2 mm, and the volume of deposits was 20 µl.

For the analyses carried out by HPLC, the method used was described by Miet and Karmas (1978) [13]. The elution system is isocratic and composed of: Acetonitryle: Methanol: Bidistilled water: Acid acetic (43: 30:26: 1). The same methods of extraction and derivation (Dansylation) were used for the TLC and HPLC technics [11].

Phenotypical identification of bacterial strains:
The phenotypical identification of entreobacteria strains was made on adequate culture media and confirmed by the API20 Gallery.

Genetic characterization of bacteria:
The genetic methods used for the characterization of the strains producing biogenic amines were the ERIC-PCR (Enterobacterial repetitive intergenic consensus polymerase chain reaction) and the Reference mark-PCR (repetitive extragenic palindromic polymerase chain reaction), described by the same authors [14, 15]. For the extraction of the bacterial DNA the method used was the same as described by Somarrlli and al, 2006 [16].

RESULTS AND DISCUSSION

Isolation and genetic characterization of bacteria:

The results found showed that 9 strains are producing histamine and 6 are producing putrescine. It is noted that these bacteria belong to the group of Enterobacteria (gram negative bacteria). Indeed the sardine (fish with red flesh) vehicle more histamine producing bacteria than the wolf (fish with white flesh), explaining their implication in histamine intoxications. Several authors used other methods for the selection of the amine producing bacteria. Certain authors used technics based on the gas production and the acidity of the culture medium (pH= 5,7 ± 0,2) [1, 17]. These authors used an infusion of sardine or tuna added with corresponding amino acids, in order to produce a medium closer to the origin of bacteria. Indeed the enzymes of the production of biogenic amines are, in general, active with a pH= 5,7 ± 0,2. Thus the bacteria growing on this medium may be biogenic amine producing bacteria. Furthermore, the transformation of amino acids to biogenic amines leads to the production of the gas CO_2. However, the produced gas can be of another compound that CO_2.

In this work, we didn't record the production of gas by 4 putrecine producing bacteria and 2 histamine producing bacteria. This finding may be explained by the fact that the small quantity of CO_2 produced during the decarboxylation was solubilized in the culture medium [17]. This result confirms that the selection of the amine producing bacteria based on the production of gas is vague.

Table 1: Number of strains producing biogenic amines detected by PCR

	Number of strains isolated	Identified genes and number of strains producing biogenic amines
Sardine (*Sardina pilchardus*)	SPC : 52 Enterobacteria : 33 LAB : 25	10 strains of Histidine decarboxylase 2 strains of Ornithine decarboxylase 0 strain of Tyrosine decarboxylase
Wolf (*Dicentrarchus labrax*)	SPC : 22 Enterobacteria : 34 LAB : 26	2 strains of Histidine decarboxylase 4 strains of Ornithine decarboxylase 0 strain of Tyrosine decarboxylase

Detection of the biogenic amines by TLC and HPLC:

According to the genetic results, 9 bacterial strains have the gene histidine decarboxylase (Tab. 1). The capacity of this gene to be expressed by the production of histamine was studied in an infusion of sardine added with histidine:

- The results by the TLC chromatograms showed that 7 of 9 strains studied provide the functionality of their genes to produce histamine (Fig. 1). This leads us to suppose that the genes of the two other strains may not be expressed, or the quantity of the produced histamine is too low to be detected by TLC [18].

- We analyzed the products of these strains by HPLC. The results found confirmed that the 9 strains having the gene histidine decarboxylase produce variable levels of histamine, between 2500 ppm and 165000 ppm.

Furthermore, we detected with the TLC a spot of biogenic amine with the same frontal report/ratio as the standard of histamine in the negative control (non producing bacteria), and the HPLC analyses confirmed that this biogenic amine was not the histamine [18].

This result proved that the TLC remains a semi-quantitative method, but inaccurate for the detection of biogenic amines, and the HPLC as a complementary method for confirmation and indicating at the same time the presence and the rate of accumulation of biogenic amines.

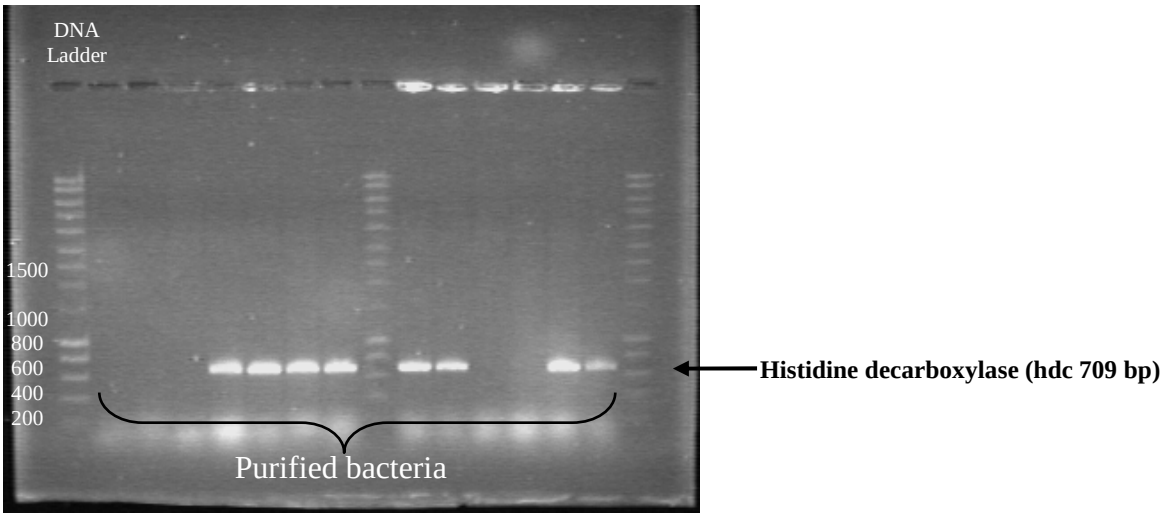

Figure 1: Detection of histidine decarboxylase with Multiplexe-PCR

The new protocol proposed in this experiment includes primarily two steps, the first step consists of the isolation of the bacterial colonies on the adequate medium, and the second of the identification of genes by PCR, the confirmation and the quantification of the amounts produced, would be done by TLC or HPLC in relation to the rapidity of production of results and the desired precision.

Comparison of the phenotypic identification and the genetic characterization of bacterial strains:
By comparing the two methods, the phenotypic method allowed the classification of our strains in 9 groups, whereas the genetic method showed the presence of 11 different groups. Among these groups, we noted that there were 3 groups, including the same bacteria in the 2 methods. Moreover, the two genetic methods used (ERIC and Reference mark) gave the same results of diversity, indicating their useful importance for the characterization of the Enterobacteria [19].

CONCLUSION

This work presents an improved method of detecting the biogenic amine producing bacteria in fish. This method is of great practical interest in avoiding histaminic intoxications, because it produces rapid and more accurate results, and it may be enlarged to other fermented and non fermented foods.

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