New molecular approaches to investigate food composition and involved microbiota

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SUMMARY

The meat food composition, the quality, the pathogenic or microorganisms involved in fermentations with a technological relevance are important focal points. Food like cheese act as substrate for the development of very complex microbial consortia in which many different microorganisms co-exist and interact in the same environment. Currently metagenomics based on New generation sequencing technology and microarray technologies represent the new frontiers respect the PCR applications. The manufacturing process of cheeses, as for most fermented food, involves a complex flora, which is composed of bacteria, yeast and filamentous fungi. The molecular approaches are direct application of molecular biology techniques in order to assess the microbial diversity without the need for cultivation (culture-independent methods). The hybridization and real-time PCR tools allow the one step identification of various species by amplifying very small DNA fragments (100–150 bp), especially in processed food. The approach is applicable to investigate adulteration in food. Other kind of food as typical cheese can be studied by molecular approach. Metagenomics is the study of organisms in a microbial community based on analyzing the total DNA within a sample. The optimization, validation and employing of the method for screening and quantitative analysis to identify the meat species in complex food mixtures represent the output of the experimentations. In our experience adulteration was found in meat products revealed by not declared animal species materials. Moreover the output of the sequence analysis on cheese obtained by NGS test, was represented by a list of various bacterial genera differently represented as species and percentage. Finally the integrated analytical protocol can permit to check the quality of food as meat and cheese monitoring the production disciplinary.

KEY WORDS

Food; human health; biota; molecular methods; DNA examination; PCR; microbial diversity.

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INTRODUCTION

Currently Food quality and safety is the main target of investigation linked to human health. Therefore, reliable paths to detect, quantify, identify, characterize and monitor microorganisms occurring in food are of great interest. Hygiene and right labeling notified on the label of any food stuff are very important criteria especially for public health. Food safety covers all the preventive measures for the delivery of food in healthy and hygienic conditions to the consumer by protecting it from denaturation, microbiological and chemical contamination and adulteration. Inadequate management of food safety causes serious health problems. There are many contaminations and infectious diseases called zoonosis transmitted from foods of animal origin.

The meat food composition, the quality, the pathogenic or microorganisms involved in fermentations with a technological relevance are important focal points. In the case of fermented foods, microorganisms play a pivotal role contributing to the improvement of the physiochemical, sensory and safety characteristics of the final products. They do not only produce organic acids that lower the pH and limit the...
growth of pathogenic and spoilage microorganisms, but also perform metabolic activities contributing to aroma development. Food like cheese act as substrate for the development of very complex microbial consortia in which many different microorganisms co-exist and interact in the same environment. The traditional microbiological analysis based on the use of synthetic culture media, is not always appropriate to study complex microbial communities because it often fails to properly profile the existing diversity. For this reason, new approaches based on the application of molecular methods have been developed. Currently Metagenomics based on New generation sequencing technology and microarray technologies represent the new frontiers respect the PCR applications. The molecular approaches are direct application of molecular biology techniques in order to assess the microbial diversity without the need for cultivation (culture-independent methods). Moreover the animal species identification detected by targeting nucleic acids extracted directly from the food sample, permits to investigate mixed meat or trace for forensic cases. Optimized molecular techniques in the field of food represent powerful tools available for the monitoring and improvement of food quality and safety. The resolution of 09/04/2003 published in OJ n. 93 of 22/04/2003 some clarification on the application procedures to improve the European Regulation on the labeling of beef and beef products. In terms of food safety related to the control of animal species, molecular techniques are currently the methods of choice to guarantee the consumers. However, when recognition of species or complex processed food is no longer recognizable, DNA examination remains the only available tool. Two experimental approaches can be considered accordingly to the original biological matrix. It is important to consider the raw material, which could either consist of a single animal specie or made with a mixtures of different animal species. DNA barcoding based on Cyt-b sequencing is generally used in the first case, whereas microarray as well as real-time PCR analysis are applied in the second case to discern among the various target animals. The hybridization and real-time PCR tools allow the one step identification of various species by amplifying very small DNA fragments (100–150 bp), especially in processed food. Not conformed samples by screening test based on microarray are confirmed and quantified by real-time PCR technology. The approach is applicable to investigate adulteration in food.

Other kind of food as typical cheese can be studied by molecular approach. The manufacturing process of cheeses, as for most fermented food, involves a complex flora, which is composed of bacteria, yeast and filamentous fungi. They can be directly inoculated as starter culture or develop from the food-chain environment. Therefore the exact composition of most cheeses is not completely known. The control of cheese products for constant quality needs a characterization of the cheese flora and a precise taxonomic identification. Metagenomics is the study of organisms in a microbial community based on analyzing the total DNA within a sample. The high-throughput DNA sequencing approaches can be used to decipher food microbial ecosystems (WHO; Mataragas et al., 2008). By detecting the bacteria species developing in the cheese it could be possible to study all the maturation steps during milk transformation and the storing in controlled environment. The integrated analytical protocol can permit to check the quality of food as meat and cheese monitoring the production disciplinary.

**Material and Methods**

For meat derived food, DNA was extracted using special kits that are based on the use of affinity columns or resins of silica-magnetite. As screening the microarray chipron technology was applied. Chipron LCD Array Kit Meat 4.0 (Chipron GmbH), is a DNA-based identification kit for animal species. With its sophisticated design of primer and probe, Meat 4.0 provides user a fast and reliable screening method to identify 24 animal species in a very short protocol. Special designed software let user receive identification report with just few clicks. To optimize the quantitative Real time test we employed serial dilutions of standard DNA for each bovine, ovine, pork and horse species in a concentration range spanning from 100% to 0.01% (w/w %). The results were automatically processed by the dedicated software. The detection limit was fixed in the order of 0.01 % w/w.

For cheese a quantity as 20 g from rind and core of 4 month Sicilian pecorino cheese was taken and homogenized in a Stomacher and extracted with Mericon kit (Qiagen) according to manufacturer’s instructions. A DNA library was prepared from total DNA using the TruSeq kit v3 (Illumina) following the manufacturer’s specifications with an average fragment size of 300 bp. The sequencing was performed on the MiSeq (Illumina) platform and an illumina pipeline was used to assess the taxonomic annotation based on 16S rRNA marker genes (Ribosomal Database).

**Results**

We analyzed with the combined approach 20
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...bacillus were more abundant. (Other species were considered as the subdominant populations, which represented more or less 10% of abundance. Unclassified genera were about 10% of genera and 45% of species. Among the non-dominant populations, some were dairy bacteria but most part of them were contaminant indicators for food sanitary quality. Different quantity and quality of bacteria species were detected trough cheese rind and core of the analysed cheese. Sequencing and assembly statistics are shown in figure 1.

DISCUSSION AND CONCLUSIONS

The microarray approach (Fig. 2) is useful as screening test to determine each animal species while Real time test permit to quantify a species specific DNA target in cases of fraud. The results obtained by DNA Microarray indicated as various kind of samples were labelled incorrectly, and adulteration was made in contrary to the notifications on the label. The adulteration was detected mostly in sausages and hamburger. It was mostly seen that meat balls and ground meat have significantly potential risk for adulteration. The inspection of the declared composition of food stuff as notified on its label is officially an obligatory task order to protect the public benefits and health against adulteration and infectious diseases caused by zoonoses. In conclusion, adulteration is a serious food safety and quality issue with an increasing prevalence in meat and meat products all over the world. Regular controls for adulteration in meat and meat products should be frequently and intensively done due to prevent the fraud risk. It was found that the indicative value obtained by DNA Microarray can be confirmed and valued by Real Time PCR assays for four animal species at moment.

For cheese metagenomic analysis the results ob-

<table>
<thead>
<tr>
<th>Classification</th>
<th>Number of Reads</th>
<th>% Total Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclassified at Species level</td>
<td>538,293</td>
<td>45.87 %</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>111,325</td>
<td>9.49 %</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>103,010</td>
<td>8.78 %</td>
</tr>
<tr>
<td>Lactobacillus uthenensis</td>
<td>79,104</td>
<td>6.74 %</td>
</tr>
<tr>
<td>Lactobacillus fermentum</td>
<td>55,538</td>
<td>4.73 %</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii</td>
<td>39,756</td>
<td>3.55 %</td>
</tr>
<tr>
<td>Streptococcus gallinarus</td>
<td>39,005</td>
<td>3.22 %</td>
</tr>
<tr>
<td>Streptococcus vestibularis</td>
<td>26,894</td>
<td>2.22 %</td>
</tr>
</tbody>
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Table 1. Food samples analysed by microarray and Real time PCR.
tained were consistent for taxonomic identification and abundance. Most of the genera detected in this work have often been described as a part of the microbiota of non-pasteurized milk too (Jonker et al., 2008; Ercolini et al., 2012). The genus Lactobacillus is commonly found in both parts of the cheese but on the rind were revealed more streptococcus respect lactic bacteria; in contrast in the core part lactobacillus were more abundant. This work is the first metagenomic report on a typical Sicilian cheese production that confirm the applicability of the method on the production chain.

For other references see Morales et al., 2011; Settanni et al., 2012; Wielinga et al., 2012; Quigley et al., 2013).

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