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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 aduleration 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 represent 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 criterias 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 coexist 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 anIllumina pipeline was used to assess the taxonomic annotation based on 16S rRNA marker genes (Ribosomal Database).

Results

We analyzed with the combined approach 20

A

Classification	Number of Reads	% Total Reads
Unclassified at Species level	538,293	45.87 %
Streptococcus thermophilus	111,325	9.49 %
Streptococcus pluranimalium	103,010	8.78 %
Lactobacillus ultunensis	79,104	6.74 %
Lactobacillus fermentum	55,538	4.73 %
Lactobacillus delbrueckii	39,729	3.39 %
Streptococcus gallinaceus	39,005	3.32 %
Streptococcus vestibularis	26,094	2.22 %

B

Classification	Number of Reads	% Total Reads
Unclassified at Species level	664,654	43.08 %
Streptococcus pluranimalium	177,495	11.50 %
Lactobacillus ultunensis	132,735	8.60 %
Lactobacillus delbrueckii	103,770	6.73 %
Streptococcus thermophilus	79,032	5.12 %
Lactobacillus fermentum	72,708	4.71 %
Streptococcus gallinaceus	34,159	2.21 %
Lactobacillus helveticus	25,054	1.62 %

Figure 1. A: Bacterial species and reads number obtained by analisys of the rind of the cheese. B: Bacterial species and reads number obtained by analisys of the core of the cheese.

Food sample	As declared	Detected DNA
Hamburgher	bovine	Bovine, turkey
Tortellini	bovine	Bovine, chicken
Sausage	Pork	Pork
Sausage	Pork	Pork
Hamburgher	bovine	Bovine, turkey
Meatballs	bovine	bovine
Meat rolls	bovine	bovine
Sausage	Pork	Pork
Salame	Pork	Pork
Meat chopped	bovine	Bovine turkey
Hamburgher	bovine	bovine
Sausage	Pork	Pork
Salame	Pork	Pork
Meat chopped	bovine	Bovine, chicken
Sausage	Pork	Pork
Hamburgher	bovine	bovine
Meatballs	bovine	bovine
Meat rolls	bovine	bovine
Sausage	Pork	Pork

Table1. Food samples analysed by microarray and Real time PCR.

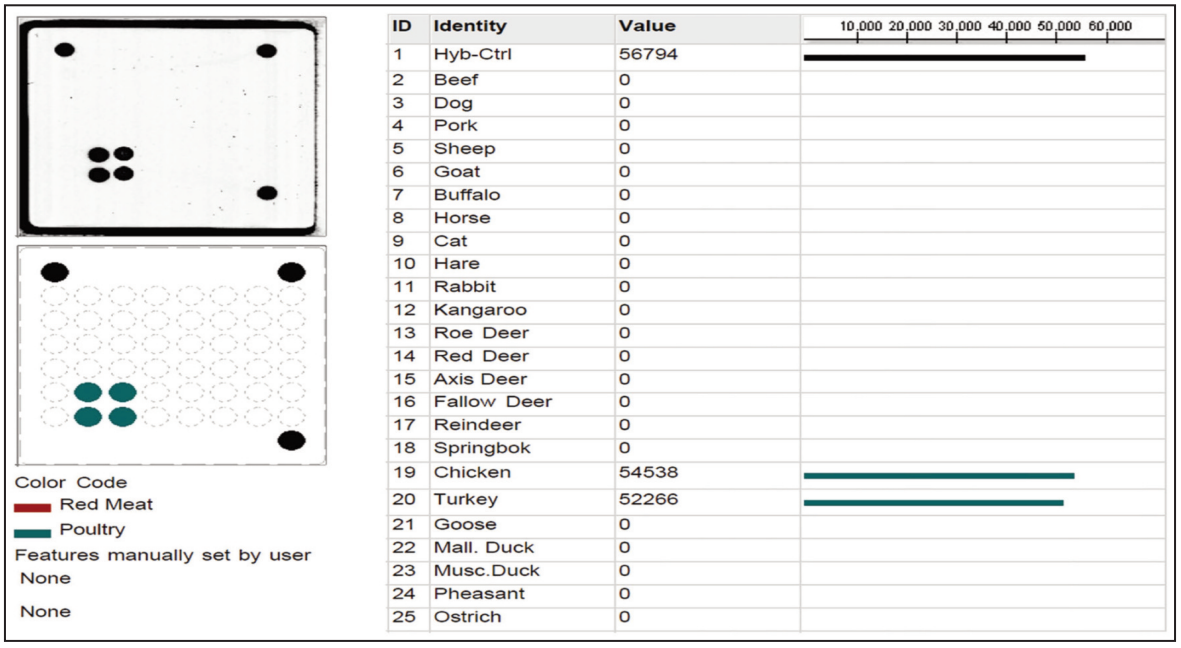
meat samples as hamburger, mixed meat, sausage, etc. The results of the experiment are given by the optimization, validation and employing of the method in terms of screening and quantitative analysis for the determination of meat species in complex food mixtures. Aduleration was found in sausage and hamburger where pork and turkey meat were revealed but not declared.

The output of the sequence analysis on cheese, was a list of various bacterial genera. In our study at least 7 different species were determined. In the esamined cheese, the two genera *Streptococcus*, *Lactobacillus* 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 determinate each animal species while Real time test permit to quantify a specie 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 prevalance 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-



ID	Identity	Value
1	Hyb-Ctrl	56794
2	Beef	0
3	Dog	0
4	Pork	0
5	Sheep	0
6	Goat	0
7	Buffalo	0
8	Horse	0
9	Cat	0
10	Hare	0
11	Rabbit	0
12	Kangaroo	0
13	Roe Deer	0
14	Red Deer	0
15	Axis Deer	0
16	Fallow Deer	0
17	Reindeer	0
18	Springbok	0
19	Chicken	54538
20	Turkey	52266
21	Goose	0
22	Mall. Duck	0
23	Musc.Duck	0
24	Pheasant	0
25	Ostrich	0

Figure 2. Microarray test on meat samples. The spots indicate the hybridization signals and the report show the quantitative index that reveal the relative abundance of each specie-specific DNA target.

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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