Short communication

Microdroplet-based multiplex PCR on chip to detect foodborne bacteria producing biogenic amines

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A B S T R A C T

The development of fast, reliable and culture-independent molecular tools to detect bacteria producing biogenic amines deserves the attention of research and ultimately of the food industry in order to protect consumers’ health. Here we present the application of a simple, low-cost, fast and sensitive method to perform microdroplet-based multiplex PCR, directly on a food matrix, for the simultaneous detection of bacterial genes involved in biogenic amine biosynthesis. After inoculating wine with Lactobacillus brevis IOEB 9809, cell lysis and DNA amplification are performed in one single step, without preliminary nucleic acid extraction or purification treatments. The assay is performed in about 30 min, requiring 150 nL of starting sample and it enables the detection of down to 15 bacterial cells. With respect to traditional culture techniques, the speed, the simplicity and the cheapness of this procedure allow an effective monitoring of microbial cells during food-making and processing.

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1. Introduction

Controlling food quality and safety, from primary production through processing and storage, to transportation and marketing, is crucial to reduce the risk of illness and guarantee the protection of consumers’ health. Special attention is required for fermented foods or for food undergone inappropriate processing and storage conditions. Wine, cheese, beer, sausages and yeast extracts can contain bacteria responsible for the production and accumulation of biogenic amines (BAs) (Russo et al., 2010; Beneudce et al., 2010).

BAs are basic nitrogenous compounds with low molecular weight, usually produced by lactic acid bacteria (LAB) through amino acids decarboxylation or deamination (Silla-Santos, 1996; Spano et al., 2010). However, in food such as fermented sausages, BA are also produced by genera of the family Enterobacteriaceae, such as Citrobacter, Klebsiella, Escherichia, Proteus, Salmonella and Shigella (Suzzi and Garnini, 2003). The main BA found in food and fermented beverages are histamine, tyramine, putrescine and cadaverine (Landete et al., 2007). In higher animals these compounds are involved in many biological and physiological processes, such as synaptic transmission, blood pressure control, regulation of body temperature and allergic response (Russo et al., 2010). Nevertheless, the ingestion of food containing high amounts of BAs can induce several toxicological effects. For example, histamine causes headache, edema, diarrhea, allergy, respiratory difficulties; tyramine can induce hypertension, migraine and neurological disorders; while putrescine and cadaverine may have carcinogenic effects (Shalaby, 1996; Ladero et al., 2010). In alcoholic beverages such as wine and beer the toxic effects of BAs are aggravated by the presence of ethyl alcohol, which inhibits the detoxification activity of diamine oxidase in human gut, causing gastric acid secretion, tachycardia and high blood pressure (Shalaby, 1996).

Overall, the potential toxicity of BAs and their use as indicators of microbiological contamination have driven the development of analytical methods for their rapid determination in food matrices.

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Among the methods for the detection of BA-producing bacteria, several are based on conventional culture methods, which are often labor and time consuming, and of low sensitivity and accuracy, since they often give false positive/negative results. Furthermore, they often enable the detection of only a BA-producer (Landete et al., 2007, 2011). On the contrary, molecular methods, especially based on the PCR technique, represent a valid alternative to traditional culture methods, being preferable in term of speed, sensitivity and reliability. In fact, PCR has successfully been used to detect BA-producing bacteria, by the specific amplification of genes encoding decarboxylase enzymes, with the unique advantage of detecting the potential BA production in a given sample before the amines themselves are synthesized (Landete et al., 2005; Lucas et al., 2005). Moreover, by using multiple target genes, the polymerase chain reaction (PCR) technique allows the simultaneous identification of bacteria producing different BAs, present in the same sample (Coton et al., 2010; Coton and Coton, 2005; de las Rivas et al., 2005).

So far, one of the greatest drawbacks in the detection of BA-producing bacteria remains the analysis on real food matrices. A preliminary treatment is usually required to remove potentially interfering compounds from the whole sample or to increase the recovery of detectable BA-producing bacteria (Onal, 2007). Therefore, there is a need to develop new, easier, more sensitive and less time-consuming methods for the determination of BA-producers directly in food. A useful tool to tackle this issue is represented by microfluidic systems (Beard et al., 2004), which offer numerous advantages such as short analysis time, reduced volumes of biological samples used, high degree of miniaturization and integration of different processing modules (Zhang and Ozdemir, 2009; Neethirajan et al., 2011; Zhang et al., 2006, 2011).

Here we propose a simple, fast and sensitive on-chip approach to detect, for the first time, BA-producing cells, directly in wine, chosen as model of a real food matrix, by multiplex PCR based on microdroplets. The molecular assay was addressed to determine two genes involved in the bacterial production of tyramine via tyrosine decarboxylase and putrescine via agmatine deiminase, respectively. The here described method showed higher efficacy and potential with respect to approaches conventionally used to detect BA-producing bacteria, such as cell culture (differential or enzymatic growth methods as well as flow cytometry; Landete et al., 2011) or molecular indirect methods (hybridization, sequencing, fingerprint; Ivey and Phister, 2011). In fact, the proposed microfluidic device offers numerous advantages in terms of fast analysis, small used volumes and high sensitivity. Moreover, the specific amplification of genes encoding tyrosine decarboxylase and agmatine deiminase enzymes allowed us both to establish the presence of BA-producers thus determining the potential BA production in a given sample before the amines synthesis (Landete et al., 2011; Lucas et al., 2005) and to simultaneously identify genes involved in different BAs biosynthesis in the target sample. To our knowledge, we employ, for the first time, a PCR microfluidic device for monitoring BAs in a microdroplet of food matrix.

2. Materials and methods

Bacterial cell lysis and multiplex PCR were performed by means of a microfluidic device operating in flow-oscillating modality and constituted by a glass microchannel embedded in an elastomeric matrix of polydimethylsiloxane (PDMS), as previously reported (Polini et al., 2010; Sciancalepore et al., 2011). The chip design was further improved and simplified by integrating a single microfabricated Ti/Pt heater underneath the capillary (instead of three different heaters as in the previous chip configuration), allowing to control the temperature needed for DNA extraction and amplification. Moreover, to avoid surface adsorption of PCR mix components, the internal wall of the glass microchannel was pretreated with a solution of 5% v/v of dimethylidichlorosilane in heptane. The chip used a two-phase liquid system consisting of a microdroplet (1 μL) of biological sample in a continuous phase of silicone PDMS oil with viscosity of 500 cP.

For testing the device performances in detecting genes involved in BA production 100 mL of Negroamaro wine were sterilized by filtration and the filtrate inoculated with 10⁵ CFU/mL of Lactobacillus brevis IOEB 9809 (Lucas et al., 2007). Then, the inoculated samples were incubated at 25 °C for 10 days and a 5 mL aliquot was centrifuged, finally the pellet was suspended in 50 μL of sterile deionized water (Pathmanathan et al., 2003; Torija et al., 2010). We focused on the extraction of tyrosine decarboxylase (tyrdc) and agmatine deiminase (agdi) genes, involved in the production of tyramine and putrescine, respectively. The simultaneous detection of tyrdc and agdi genes was obtained by combining two sets of primers (Primm srl, Milan, Italy): TDC_F (5′-TGAAGAGGGTG CCGATAATC-3′) and TDC_R (5′-GCCCTTC CAATTCCTCA-TA-3′), amplifying a product of 141 bp, for tyrdc gene fragments; AGUA1_F (5′-CTCTGATAATGCGACAGA CG-3′) and AGUA1_R (5′-TCCAGG TAGCCGAGT TTT-3′), amplifying a product of 240 bp, for agdi gene fragments. Primer concentrations were 0.35 μM for TDC_F and TDC_R and 0.4 μM for AGUA1_F and AGUA1_R. 1 μL reaction mixture contained ultrapure water, reaction buffer [160 mM (NH₄)₂SO₄, 670 mM tris-HCl (pH 8.8), 0.1% v/v Tween 20] (Euroclone, Milan, Italy), 1.5 mM MgCl₂ (Euroclone), 0.2 μM of each dNTP (Euroclone), 0.04 units of Taq Tag DNA polymerase (Clontech, Mountain View, USA) and 150 nL of biological sample. Ultrapure water was used as negative control instead of the DNA sample.

For lysis and amplification experiments, a 1 μL droplet of sample was introduced into the capillary and was initially heated at 95 °C for 5 min. Then, a two-step PCR was performed, where the annealing and extension steps of the thermal profile were combined in a unique phase. Specifically, on-chip optimized protocol consists of (i) hot start at 95 °C for 1 min, (ii) 35 cycles, each composed by 15 s at 95 °C, and 30 s at 60 °C, (iii) final extension at 60 °C for 1 min. For temperature cycling, the microdroplet was moved in an oscillating way within the oil phase from the denaturation to the annealing/extension region. The direction of the fluid motion was changed after each denaturation and annealing/extension step. The PCR was performed on 4 serial dilutions of the bacterial pellet suspension in double-distilled water, namely1:50, 1:100, 1:500, 1:1000. The PCR products were analyzed by electrophoresis on 1.5% agarose gel after ethidium bromide staining and visualized by ultraviolet illumination. Thin-layer chromatography (TLC) analysis for detection of tyramine and putrescine was performed as already described (Romano et al., 2012).

3. Results and discussion

The here proposed microfluidic system, employed to detect BA-producing bacteria directly from a wine sample, offers many advantages in terms of device architecture and fluidic modality. In fact, microfluidic chips based on capillaries encased within PDMS (Li et al., 2010; Zhai et al., 2007) and smart tubes (Dimov et al., 2010) allow the construction of rapid and easy-to-use diagnostic platforms. In particular, using microfluidic channels made of glass offers great fabrication simplicity, good thermal stability, reduced or no sample evaporation during the analysis, blocking vapor diffusion that normally occurs through bare PDMS devices (Polini et al., 2010). Moreover, the flow-oscillating mode allows us to have high flexibility in the number of thermal PCR cycles, and to potentially parallelize multiple reactions (Sciancalepore et al., 2011).

A schematic representation of the reaction site into the chip is shown in Fig. 1a, which displays a micrograph of the microdroplet.
The result demonstrated the great efficiency of the developed method, with no appreciable reduction in the reaction yield even with the lowest sample concentration. In details, the high performance of the chip led to saturation the amplification product, thus achieving a comparable result for the different dilutions tested. Moreover, the obtained amplification results revealed the capability of the on-chip process both to combine bacterial cell lysis and multiplex PCR in one single step and to efficiently detect 15 BA-producing cells directly in a liquid food matrix, without any preliminary nucleic acid extraction (Coton et al., 2010; de las Rivas et al., 2005; Pinzani et al., 2004) or sample treatments (Coton and Coton, 2005).

The total time needed for completing the reaction was 32 min, much faster than standard PCR assays typically reported for detecting BAs (requiring more than 1–2 h) (Coton et al., 2010; Onal, 2007; de las Rivas et al., 2005). In fact, the application of a two-step PCR guarantees remarkable time saving and simpler experimental operation with respect to a traditional three-step reaction, in which three different temperatures are used for PCR cycles. In comparison to a standard thermal cycler, we obtained a remarkable reduction of

in the oil phase, totally positioned on the heater, thus guaranteeing a uniform sample heating. We carefully calibrated the temperature distribution along the central axis of the microfluidic channel by using a thermocouple with an external diameter of 0.25 mm, which keeps constant the temperature of the fluid above the heater at 95 °C. As displayed in Fig. 1b, a thermal gradient of about 7 °C/mm is present along the capillary, due to convective heat transfer from the heater (Sciancalepore et al., 2011). During the device operation, the region nearby the heater was used for DNA extraction and denaturation, whereas a microchannel region, 5 mm away from the heater, was devoted to the annealing/extension step (gray rectangles in Fig. 1b). In this way, only one heater was required to obtain the desired PCR thermal profile, reducing considerably the complexity and the cost of the device and making it especially attractive for bio-organic laboratories and agro-food applications.

Before performing the microfluidic PCR reaction, the capability of the L. brevis IOEB 9809 to produce BAs in wine was confirmed by thin layer chromatography (TLC) analysis (Romano et al., 2012). To this aim, we inoculated wine with L. brevis and then a sample was analyzed by TLC assay (Romano et al., 2012; Arena et al., 2011). The BAs produced by L. brevis strain were compared with an internal standard, consisting of four purified BAs (tyramine, histamine, cadaverine, putrescine). The TLC analysis (Fig. 2) confirmed that L. brevis IOEB 9809 produces both tyramine and putrescine, two of the main BAs detectable in the most common fermented foods, such as wine and cheese (Spano et al., 2010).

Furthermore, the presence of tyrdc and agdi genes was analyzed performing both cell lysis and DNA amplification in one single step into the microfluidic platform. The sample was first pre-heated at 95 °C for 5 min, then a two-step PCR was performed, combining the annealing and extension steps of PCR thermal profile in a unique phase. We established that the thermal pretreatment of the sample is strictly required and fully effective for breaking bacterial cell walls and releasing DNA. In fact, we verified, as negative control, that without such early heating stage no PCR reactions take place even in standard thermocyclers (not shown). Fig. 3 shows the specific products from the microdroplet-based amplification of tyrdc and agdi genes. In order to assess the sensitivity of the on-chip method, we tested four serial dilutions of the bacterial pellet suspension in double-distilled water, namely 1:50 (Fig. 3a), 1:100 (Fig. 3b), 1:500 (Fig. 3c) and 1:1000 (Fig. 3d), corresponding to 300, 150, 30 and 15 bacterial cells, respectively. The result demonstrated the great efficiency of the developed method, with no appreciable reduction in the reaction yield even with the lowest sample concentration. In details, the high performance of the chip led to saturation the amplification product,
the PCR run time (32 vs. 50 min), which was also related to the used flow-oscillating modality, since sample heating/cooling rates were three times faster (16°C/s instead of 5°C/s) by virtue of the lower thermal inertia (Sciancalepore et al., 2011).

4. Conclusions

In spite of the growing research interest on the negative impact of BA on human health (Shalaby, 1996; Ladero et al., 2010), a shared legislation on the maximum concentration of BAs in food is still lacking. Only recently the European Community has started to define the critical levels of some BAs in foodstuff (Russo et al., 2010; Spano et al., 2010). In this framework, food monitoring to detect BA-producing bacteria is of great relevance not only for their potential hazard to human health, but also for economic issues, since products containing BA concentrations higher than recommended limits can be rejected in commercial transactions. The here presented on-chip analytic approach can be exploited in several food analysis, potentially covering all the phases of food production and commercialization because of its low costs and high portability, and addressing the challenge of the zero-tolerance level even for pathogenic microorganism detection (Batt, 2007). Furthermore, it is adaptable to spoilage or pathogenic bacteria, with potentially widespread application in food monitoring as well as in molecular biology, clinical and forensic laboratories.

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