Trends in Food Science & Technology 46 (2015) 295–301

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: http://www.journals.elsevier.com/trends-in-food-science-and-technology

Microbiome profiling in fresh-cut products

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ARTICLE INFO

Article history:
Received 29 December 2014
Received in revised form
13 October 2015
Accepted 18 October 2015
Available online 20 October 2015

Keywords:
Metagenomics
Microbiome
Fresh-cut products
High throughput sequencing

ABSTRACT

Fresh-cut fruits and vegetables are highly perishable and minimally processed products. One of the main issues related to their safety is the potential microbial contamination and spoilage that can significantly reduce their shelf-life. To tackle this problem, the complete identification of fresh-cut product microbiota is of primary importance. Indeed, despite the huge amount of metagenomics data generated in the last decade to profile food microbiome, a specific knowledge about fresh fruits and vegetables microbiome is still scarce. Production of additional food-related data, such as improved microbial genome assemblies and increased number of microbial reference genome sequences, are needed to perform a robust and reliable analysis of food-related metagenomics data. These data can be successfully applied to model the contribution of individual microorganisms for the quality and safety of fresh-cut products.

1. Introduction

The market of fresh-cut products has witnessed a dramatic growth in recent years, largely stimulated by consumer demand for fresh, healthy, convenient and additive-free foods. In 2010, fresh-cut products outlined 4% of the total volume of the EU vegetable market and 1% of the EU fruit market. At the same time, fresh-cut salads and fresh-cut fruits reached 50% and 10% of the EU fresh-cut markets volume respectively (source Euromonitor).

Fresh-cut products are highly perishable, subjected to rapid microbial spoilage (short shelf-life) and, in some cases, to pathogen contamination.

Spoilage is one of the major causes of economic loss for the fresh-cut product industry (Gram et al., 2002) and recent studies have identified several bacterial species, belonging both to gram-negative and gram-positive bacteria, as spoiler organisms in different foods (Nieminen et al., 2012; Remenant, Jaffrès, Dousset, Pilet, & Zagoreca, 2015). In addition to spoilage problems due to microorganism, fresh-cut products are “minimally processed” and their pathogen contamination can occur during different phases of the food-chain (Francis et al., 2012). In the last decade, the Center of Disease Control and Prevention associated fresh fruits and vegetables with 6–8% of the food borne disease outbreaks in U.S. (Painter et al., 2013). The most relevant foodborne pathogens in fresh-cut products are *Salmonella* spp., *E. Coli*, Campylobacter spp., *Shigella* spp., *Clostridium* spp., Staphylococcus aureus, Yersinia spp. and Cyclospora spp.. However, the incidence of outbreaks of food borne viral disease such as Norovirus and *Hepatitis* A virus has increased considerably in the last decades (Authority EFS, 2015; Callejón et al., 2015; Herman, Hall, & Gould, 2015).

Despite fresh-cut products are more subjected to pathogen contamination than processed food, the microbiota of the former is rich in strains with natural antagonist properties against pathogens and phytopathogens. The study of the natural biodiversity of microbial communities is also related to the need of a more detailed information on the microbial/plant interactions.

The microbial consortia can contain both beneficial and phytopathogenic microorganisms which have a significant influence on plant health and food quality. In the last few years, Ottesen et al. (2013) investigated the tomato microbiome and its role in food safety, and grapevines microbial communities and the influence of the latter on vine performance and wine quality was investigated by Pinto et al. (2014). It is also known that autochthonous lactic acid bacteria isolated from fruits and vegetables can have a probiotic potential for human and inhibit food pathogens (Vitali et al., 2012). Moreover, many studies reported that Lactobacillus strains are able to produce antimicrobial molecules that improve food safety (Gálvez, Burgos, López, Pulido, & Springer, 2014). Recently, *L. plantarum* and *L. paracasei* strains were isolated from fresh lamb's lettuce and apple and used as antimicrobials to enhance food safety and extend the shelf-life of the ready-to-eat products (Siroli et al., 2015).

It is also appropriate to mention the increasing importance of plant virome, that can cause both plant and human foodborne

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http://dx.doi.org/10.1016/j.tifs.2015.10.013
0924-2244/

illness (Rodriguez-Lazaro et al., 2012; Valkonen, 2014). The study of plant viruses is relatively new but evidences suggest that these viruses can impact on human health (Colson et al., 2010). Recently, many authors (Park et al., 2011; Stobbe & Roossinck, 2014) reported that plant virus diversity is still underestimated and there is a lack of knowledge that needs to be filled to better evaluate the food safety.

In order to control and minimize the microbiological hazard of food borne pathogens, as well to enhance food products shelf-life, food microbiome needs to be identified in a rapid and unequivocal way. The aim of this review is to provide an overview of the main technologies used in the last decade to profile the microbiome in fresh-cut fruits and vegetables.

2. Microbial identification methods

Food microbiological analysis is integrated part of the safety management in the food chain. The monitoring and control of food microbiota is mainly carried out by classical methods based on culture-dependent approaches. However, during the last ten years many efforts have been made to introduce and validate DNA-based methods (Ceuppens et al., 2014).

2.1. Classic methods

Bacterial species are identified by culture in specific growth media and the primary classification is based on phenotypic characteristics such as colony morphology, growth conditions, Gram staining and biochemical tests (Hemraj, Diksha, & Avneet, 2013).

Classic identification methods are widely used despite some disadvantages. Apart from being slow and laborious, they are useful only if applied to organisms that can be cultivated in vitro or previously characterised. Furthermore, some strains exhibit unique biochemical characteristics that do not fit into any traditional patterns used to characterize known genus and species, as recently occurred for the pathogenic strains *E. Coli* (Alqasim, Scheutz, Zong, & McNally, 2014; Brzuszkiewicz et al., 2011) and *Yiersinia* (Reuter et al., 2014).

Finally, it is well known that many non-bacterial species (e.g. fungi, viruses) are particularly present in food and in minimally-processed food such as fresh-cut products. The identification and cultivation of non-bacterial species by classic microbiology tools is quite difficult (Guarro, Gené, & Stchigel, 1999; Seymour & Appleton, 2001) due to the need of specific growth conditions and symbiosis with other microorganisms. Recent outbreaks proved again that classic approaches are not able to quickly identify microbial strains and a wealth of information is still missing (Newell et al., 2010).

2.2. Culture-independent methods

In the last decades, some methodologies such as ELISA or PCR coupled with important advances in bioinformatics tools proved to be more rapid and sensitive than the classic microbiological identification protocols (Singh, Prabha, Rai, & Arora, 2012). A standard tool for bacteria identification is the 16S rRNA gene sequencing by using universal primers that amplify regions with variable sequence (Klindworth et al., 2012).

The 16S rRNA gene has been identified in a large number of species and sequence information is available in public DNA databases such as GenBank and EMBL. Using this information, the identification of an unknown strain is made by comparison of its 16S rRNA gene sequence with publically available sequences, and the closest strains are identified using in-silico analysis (e.g. BLAST). For non-bacterial species the standard region used is the Internal Transcribed Spacer (ITS) located between the 16S/18S and the 23S/28S subunit rRNAs (Van Der Vossen & Hofstra, 1996). In this case the strain identification is more difficult, due to the lack of comprehensive reference databases.

At present, there are two main nucleic acid-based and culture-independent approaches used to analyse microbiome: fingerprinting (e.g. T-RFLP and ARISA) and sequencing (e.g. high throughput sequencing). The first approach is cheap, quick and can provide a general profile of all bacterial or fungal strains present in the sample, but is not able to precisely identify them (Bulgarelli, Schlaeppi, Spaepen, van Themaat, & Schulze-Lefert, 2013). The second approach is more expensive and time-consuming, but it is able to identify, at the species level, most of the microorganisms in a microbial population (Müller & Ruppel, 2014).

2.2.1. T-RFLP and ARISA

Terminal restriction fragment length polymorphism (T-RFLP) is a DNA-based technique that uses PCR amplification of 16s/18s rRNA genes to obtain the fingerprint of the microbial community in a sample (Blackwood, Marsh, Kim, & Paul, 2003; Dickie & FitzJohn, 2007; Liu, Marsh, Cheng, & Forney, 1997; Marsh, 1999). On the other hand, Automated Ribosomal Intergenic Spacer Analysis (ARISA, Gurtler & Stanisich, 1996) is based on the amplification of the ITS region, which is variable in length and nucleotide sequence among different bacterial and fungal genotypes (Guasp, Moore, Lalucat, & Bennasar, 2000; Rajnard et al., 2001).

Both T-RFLP and ARISA are suitable to analyse any kind of samples (e.g. water, soil, rumen, food) and provide a representation of the dominant members in microbial communities. Even if the microbiome fingerprint obtained with these tools is less informative compared to more recent technologies, they are cost effective, reproducible and robust methods, which can be applied to fruits and vegetables. These fingerprinting techniques were applied to monitor changes in rhizosphere microbiome of different fruits and vegetables after pathogen infection, antimicrobial treatments or changes in soil composition (Campisano et al., 2014; Chowdhury et al., 2013; O'Neill, Deery, Scott, & Dickinson, 2014; Tkacz, 2013). ARISA was also used as first screening analysis followed by or coupled with high throughput sequencing (Jami, Shterzer, & Mizrahi, 2014; Manter, Delgado, Holm, & Stong, 2010).

2.2.2. High throughput sequencing: metabarcoding and whole metagenome

High throughput sequencing (HTS), also known as next-generation sequencing (NGS), is a term used to describe different sequencing technologies (e.g. Illumina, Roche 454, Ion torrent, PacBio) that allow to produce DNA/RNA sequence data faster and cheaper than with the classic Sanger sequencing approach (Kircher & Kelso, 2010).

The technical principles of the different sequencing platforms and workflows of sample preparation were comprehensively reviewed before (El-Metwally, Ouda, Helmy, & Springer, 2014; Hodkinson & Grice, 2015; Knief, 2014; Morey et al., 2013). Despite differences in terms of sequencing principle, the choice of HTS method is strictly related to the aims of the specific investigation (Table 1).

Whole genome/transcriptome sequencing (shotgun) is particularly useful for virus analyses, as reviewed in plants by Roossinck (2012), and for the identification of novel enzymes (Berlec, 2012). The analysis of unidentified microbiota requires de-novo sequencing and long reads could be useful to help the sequence assembly. In this case many reports underlined the advantages of single molecule method by PacBio (Koren et al., 2012; Wu et al., 2014). Finally, even if the targeted resequencing approach is, in general, less used than the shotgun, it represents a cheaper alternative to identify and confirm single nucleotide polymorphisms