Food Microbiology 54 (2016) 11–19

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Use of a nisin-producing *Lactococcus lactis* strain, combined with natural antimicrobials, to improve the safety and shelf-life of minimally processed sliced apples

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ARTICLE INFO

Article history:
Received 3 June 2015
Received in revised form
4 November 2015
Accepted 6 November 2015
Available online 10 November 2015

Keywords:
Sliced apples
Biocontrol
Natural antimicrobials
Nisin
Safety and shelf-life

ABSTRACT

The intrinsic characteristics of minimally processed fruit may favor the growth of pathogens and spoilage microorganisms. In this context, the use of natural alternatives to traditional chemical sanitizer agents may represent a useful tool to increase the shelf-life and the safety of minimally processed fruit. The aim of this study was to evaluate the application of the nisin-producing *Lactococcus lactis* CBM21 as a potential biocontrol agent in sliced apples combined or not with hexanal, 2-(E)-hexenal and citral through microbiological, colorimetric, textural and sensory assessments.

The use of *L. lactis* CBM21 limited the growth of yeasts on apples below 5 log cfu/g during 28 days of storage. Moreover, this strain significantly increased the safety of the products, inhibiting the growth of *Listeria monocytogenes*, especially when used in combination with the proposed natural antimicrobials. No negative effects on color parameters were observed after 14 days of storage in presence of the natural antimicrobials. Furthermore, the addition of the biocontrol agent was positively perceived by panelists for product flavor and odor.

Even if further studies are necessary, these results suggest that the combination of the considered "hurdles" can represent a new strategy to prolong shelf-life and ensure the safety and quality of sliced apples.

1. Introduction

The demand for minimally processed fruits and vegetables has incessantly increased in the last years reflecting the interest of consumers for fresh and healthy products with an easy way of preparation (Allende et al., 2006). The intrinsic characteristics of ready-to-eat fruits, such as the low acidity and high humidity, together with the high number of cut surfaces, may favor the growth of spoilage microorganisms and pathogens (Beuchat, 2002). These products have been implicated in outbreaks of foodborne infections caused by human pathogens, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Alegre et al., 2010; Beuchat, 2002; Lanciotti et al., 2004). Consequently, the use of raw materials of good quality and efficient decontamination procedures are critical steps to ensure the safety of ready-to-eat fresh fruits and vegetables (Gil et al., 2009; Francis et al., 2012). The resistance of some pathogenic strains toward traditional sanitizers has justified the search for substitutes to guarantee the food safety and quality. In this context, the use of biocontrol agents fits well with this new trend, and various microorganisms have been proposed as bioprotective agents (Russo et al., 2014; Vermeiren et al., 2004). Numerous studies have shown the good potential of several microorganisms to inhibit the growth of foodborne pathogens in minimally processed fruits and vegetables (Bennik et al., 1999; Leroy et al., 2003; Vescovo et al., 1996). The use of biocontrol agents, such as *Candida* spp., *Gluconobacter*

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http://dx.doi.org/10.1016/j.fm.2015.11.004
0740-0020/

spp., *Discosphaerina* spp. and *Metschnikowia* spp., have been reported to inhibit the growth of *L. monocytogenes, E. coli* and *Salmonella enterica* in fresh-cut apples, but negative effects, such as browning of fruits, have been observed (Leverentz et al., 2006). Scolari and Vescovo (2004) and Torriani et al. (1997) showed the potential of a strain of *Lactobacillus casei* to increase the safety of minimally processed vegetables due to the inhibition of *Aeromonas hydrophila*, *Staph. aureus*, *E. coli* and *L. monocytogenes*. However, literature data do not exhaustively explain the effects of biocontrol agents on the spoilage microbiota and more generally on the shelf-life of products. For this reason, there is still a need for new bioprotective microorganisms that fulfill the desired characteristics, such as biosafety and limitation of non-target effects (Trias et al., 2008).

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) by the USA Food and Drug Administration (FDA), and their use to preserve (through fermentation) meat and dairy products and to bioprotect fermented vegetables is well documented (Ruiz-Barba et al., 1994; Stiles and Holzapfel, 1997). Moreover, the capability of LAB to produce bacteriocins and other antimicrobial molecules, and their general acceptability in foods, make them interesting alternatives to chemicals in food preservation. Several authors have reported the good potential of bacteriocins to inhibit Gram-positive bacteria both in model and in real food systems, such as cheese, meat and ready-to-eat vegetables (Jamuna et al., 2005; Loessner et al., 2003; Molinos et al., 2005). Nisin was the first characterized bacteriocin and it is produced by *Lactococcus lactis* and in European Union it is allowed in some application as food preservative (FDA, 1988; Jones et al., 2005). In fact, nisin, and in particular the natural variant Z, is commonly used in various food products in order to increase their microbiological safety, due to its high solubility and stability (De Arauz et al., 2009). The activity of nisin against Gram-positive bacteria, and particularly *L. monocytogenes*, is well-known both under laboratory conditions and in foodstuffs (Cleveland et al., 2001; Yang et al., 2012). Recent studies have shown synergistic effect of nisin when used in combination with other food additives, such as sodium lactate, citric acid, phytic acid, potassium sorbate and H_2O_2 in fresh cut lettuce and minimally processed fruits and vegetables (Bari et al., 2005; Ukuku et al., 2005).

Regarding other food additives, several studies have also shown the good potential of essential oils and their principal components to increase the safety and shelf-life of minimally processed fruits both in model and food systems (De Azeredo et al., 2011; López-Gálvez et al., 2009; Siroli et al., 2014). In particular, it is well known the wide spectrum of antimicrobial activity of citral, a mixture of the 2 isomers geranial and neral, and component of several citrus essential oils, as well as of hexanal and 2-(E)-hexenal, which are components of the aroma of many fruits and vegetables (Belda-Galbis et al., 2013; Kubo and Fujita, 2001; Wuryatmo et al., 2003). In addition, their antimicrobial efficacy has already been experienced in fruit-based salads in syrup (Belletti et al., 2008), fruit-based soft drinks (Belletti et al., 2007) and minimally processed sliced apples (Corbo et al., 2000; Serrano et al., 2008; Siroli et al., 2014). In particular, Siroli et al. (2014) showed that the addition of 2-(E)-hexenal in combination with hexanal or citral, at a concentration of 125 mg/L, in the dipping solution of minimally processed sliced apples packaged in modified atmosphere had a positive effect on the product, due to their antimicrobial activity against naturally occurring spoilage species, allowing the extension of shelf-life up to 35 days, without detrimental effects on safety and quality parameters.

In this perspective, the first purpose of the present research was to identify and characterize nisin-producing *L. lactis* strains and evaluate the potential application of a selected nisin-producing strain of *L. lactis* in minimally processed apples, combined or not with other "hurdles", such as hexanal, 2-(E)-hexenal and citral, to inhibit the microbial growth. To assess their effects on product safety, challenge tests with *L. monocytogenes* and *E. coli* were also performed.

2. Material and methods

2.1. Natural antimicrobials

The tested compounds hexanal, 2-(E)-hexenal and citral were purchased from Sigma–Aldrich (Milano, Italy). These antimicrobials were selected on the basis of their antimicrobial activity and their organoleptic compatibility with sliced apples (Siroli et al., 2014).

2.2. Microbial strains and growth conditions

The strains used in this study are listed in Tables 1 and 2. *Lactococcus lactis* strains (Table 1), all isolated from dairy products, except one isolated from minimally processed apples, belong to the collection of the Department of Biotechnology of Verona University. The strains listed in Table 2 belong to the Department of Agricultural and Food Sciences of Bologna University.

Before each trial, the *L. lactis* strains were preliminarily grown in M17 broth incubated aerobically at 30 °C for 24 h; the other LAB strains were grown in de Man Rogosa and Sharpe (MRS) broth at 37 °C, 24 h; pathogenic strains in Brain Heart Infusion broth (BHI) at 37 °C, 24 h. Finally, *S. cerevisiae* cells were grown in Sabouraud Dextrose broth (SAB) at 37 °C for 48 h. All these media were purchased from Oxoid Ltd (Basingstoke, UK) and utilized according to manufacturer's instruction.

2.3. Screening for nisin-producing Lactococcus lactis strains

The 30 strains of *L. lactis,* (Table 1) were screened for their capability to produce nisin. The *L. lactis* strains was pre-cultured as reported above, then an agar spot test, following the method reported by Schillinger and Lucke (1989) was used to verify the antimicrobial activity against *L. plantarum* ATCC 14917^T, which was shown to be sensitive to nisin (Rossi et al., 2008). The cultures showing an inhibition zone larger than 2 mm around the spot were considered in the subsequent steps.

Total genomic DNA was extracted from microbial cells and purified using the Wizard Genomic Purification Kit (Promega corporation, Madison, WI, USA), following the manufacturer's recommendations.

The primers reported by De Vos et al. (1993) (forward: 5′-CGC GAG CAT AAT AAA CGG CT-3′; reverse: 5′-GGA TAG TAT CCA TGT CTG AAC-3′) were employed for the amplification of the nisin-encoding gene. The PCR mixture (50 μl) was composed by 2 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of deoxyribonucleotide triphosphates (dNTPs), 0.02 U/μl *Taq* polymerase, 1 × PCR buffer and approximately 20 μg of genomic DNA. Thermocycling conditions were: preliminary denaturation at 94 °C for 5 min; 30 cycles at 93 °C for 2 min, 54 °C for 1 min, 72 °C for 1.5 min, then a final extension at 72 °C for 10 min. PCR products were visualized on 1.5% agarose gel. The PCR resulting amplicons were purified with the QIAquick PCR Purification Kit (Qiagen, USA) and sequenced at BMR Genomics sequencing centre (Padua, Italy). Sequences were then compared with those available in GenBank database retrieved through BLASTn searches and then aligned using the GeneDoc 2.7 software.