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Nisin application delays growth of *Listeria monocytogenes* on fresh-cut iceberg lettuce in modified atmosphere packaging, while the bacterial community structure changes within one week of storage

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ABSTRACT

Listeria monocytogenes poses a risk to minimally processed ready-to-eat foods such as lettuce due to its ability to grow under refrigeration conditions. Since many natural anti-listerial products render Iceberg lettuce unsuitable for consumption within 2 d of storage, this study investigated the efficacy of Nisin A as anti-listerial agent and its sensory impact on lettuce. In addition, the evolution of the bacterial community on fresh-cut lettuce was monitored for the duration of storage. *In-vitro* assays confirmed the efficacy of Nisin A to inhibit growth of a three strain mix of *L. monocytogenes* in model atmospheres and air. The *L. monocytogenes* strain mix was added to lettuce that was subsequently treated either with Nisin, *L. lactis* DSM20729 (a Nisin A producer) or was kept without inoculation. Incubation took place at 4 and 8 °C under various atmospheres. On days 0, 2, 5 and 7, *L. monocytogenes* was enumerated on selective agar and a sensory panel graded the lettuce on visual appearance. At 4 and 8 °C a 10 to 100-fold reduction of *L. monocytogenes* growth was achieved with 5 mg kg^{-1} Nisin over a seven-day period, while lettuce kept an acceptable sensory appearance over the first 5 d. Direct application of *L. lactis* had no detectable effect on *L. monocytogenes* growth *in situ*. The bacterial community structure changed substantially from each sampling day to the next over the seven days of incubation. However, Pseudomonadaceae with the genus *Pseudomonas* were most abundant at all times and increased in relative abundance to over 90% by day 7. In conclusion, the application of Nisin A to minimally processed vegetables like lettuce seems to be a viable alternative to reduce and delay growth of pathogen *L. monocytogenes*, while not impacting the sensory appearance for 2–5 d.

1. Introduction

Production in the global fresh fruit and vegetable industry has increased in the first half of this decade by almost 40% (FAOSTAT - Food and Agriculture Organization of the United Nations (FAO, 2017). A central part of this increase is the rise in consumption of foods that are minimally processed and alongside with it food related illnesses (Omac et al., 2015). Most fresh fruit and vegetables harbour considerable amounts of natural microbes, some for which have the potential to be pathogenic such as *Listeria monocytogenes* (Francis et al., 1999). The food industry that includes fresh cut fruit and vegetables minimizes contamination through its use of chlorine washes, low storage temperatures, modified atmospheres (Francis and O'Beirne, 1997) and the adherence to the Hazard Analysis Critical Control Point (HACCP) procedure. Despite these efforts, *L. monocytogenes* is still a common occurrence (Leong et al., 2017) and is estimated to be responsible for about 260 food-related deaths each year in the USA alone (Centers for Disease Control and Prevention - CDC, 2016).

L. monocytogenes is able to grow in adverse environmental conditions, all while being regularly detected in soil, water, vegetation, livestock, food processing and storage facilities (Harris et al., 2003). Its ubiquitous presence results in easy entry into the food processing chain at various points, where it is able to survive for extended periods despite sanitation efforts (Leong et al., 2014). The contamination of fresh-cut produce that support *L. monocytogenes* growth are of particular concern as control methods including refrigeration and modified atmospheres (MAP) may be inefficient to inhibit growth over the course of storage (Scollard et al., 2016). Even lowest levels of contamination may result in multiplication beyond the limits that are considered to be safe for consumption (McManamon et al., 2017). The use of chlorine

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based products as anti-microbial treatment on fresh cut produce has raised safety concerns due to the formation of halogenated carcinogenic by-products (Ölmez and Kretzschmar, 2009; Zhang and Farber, 1996).

The application of essential oil based natural anti-listerial agents on fresh-cut fruit appeared to be a viable alternative to chlorine dipping, but their application on fresh-cut lettuce resulted in product browning beyond acceptance levels (Scollard et al., 2016). The antimicrobial peptide Nisin has been used in dairy products, dressings, sauces and post fermented meats (Adams and Smid, 2003). Nisin is regarded as a safe food additive (Cotter et al., 2005; Delves-Broughton et al., 1996) and is naturally produced by *Lactococcus lactis*. The European Union has already approved the use of Nisin (E 234) to various foodstuffs such as semolina pudding and dairy products including cheese at concentration limits of 3–12.5 mg kg^{-1} (European Council, 2008). A paucity of studies looked into the use of Nisin on ready to eat vegetable products. Xu et al. (2007) showed that a dipping solution with Nisin (50 mg kg^{-1}), 0.5% citric acid and 200 mg kg^{-1} grapefruit seed extract could reduce *L. monocytogenes* on lettuce leaves by over 2 logs (CFU g^{-1}) without a negative effect on its organoleptic properties.

The aim of this study was to test the effect of Nisin onto growth of *L. monocytogenes* on fresh cut lettuce as a single anti-listerial additive at concentrations that would be in accord with current EU guidelines of Nisin as a food additive. In addition, this study aimed to characterise the bacterial community structure of fresh-cut lettuce with and without the use of Nisin and *L. monocytogenes*. The hypotheses of this study were that i) Nisin application can sustainably inhibit growth of *L. monocytogenes* on fresh-cut lettuce at concentrations used in the food industry, ii) that the application of a Nisin producing *L. lactis* strain onto fresh-cut lettuce may be an economical substitute for application of Nisin, and that iii) Nisin application and storage conditions have a pronounced effect onto the lettuce bacterial community structure.

2. Materials and methods

2.1. In vitro Nisin assay

A mini titre plate assay was conducted (24-well plate) in order to establish the effectiveness of the purified Nisin A at various quantities (0, 5, 10 and 25 mg kg^{-1}) *in vitro* under 3 different atmospheric conditions: (a) air, (b) 8 kPa CO_2, 4 kPa O_2, 88 kPa N_2 (modified atmosphere packaging, MAP) or (c) 15 kPa CO_2, 1 kPa O_2, 84 kPa N_2 (MAP). The *L. monocytogenes* mix from Section 2.3 was used for inoculation at 10^6 CFU mL^{-1}. Into each well 1000 µL of TSA broth, 1000 µL of PBS containing the appropriate amount of purified Nisin (0, 5, 25 and 50 mg kg^{-1} final concentration) and 10 µL of *L. monocytogenes* was added aseptically in triplicate. The 24-well plate was then placed into a 35 µm think orientated polypropylene (OPP) packaging bags sealed using heat sealed at the selected atmosphere as described in Section 2.4.3. The packs were then stored (incubated) at 37 °C for 48 h. At the selected time point the plates were removed from the 37 °C incubator and analysed for cell density using a spectrophotometer (VWR, USA) at 600 nm. Loops were used to streak the well contents on LSA to confirm the presence of viable *L. monocytogenes*. Individual 24-well plates were used for each of the sampling points and then discarded after reading.

2.2. Preparation of fresh-cut iceberg lettuce

Iceberg lettuce (Class I Spain) was obtained from a local vegetable supplier (Limerick, Ireland) at the day of testing and kept under refrigeration (4 °C) until processed. Outer layers and stem were manually removed using a disinfected sharp stainless steel knife. Any damaged leaves and the core of the heads were excluded. The remaining inner leaves were sliced with a disinfected sharp stainless steel knife and cut into pieces of approx. 20 mm portions of 10 g cut lettuce were transferred into 35 µm thick orientated polypropylene (OPP) packaging bags (18 x 10 cm) which had a permeability to O_2 of 5.7 nmol m^{-2} s^{-1} kPa^{-1} and to CO_2 of 19 nmol m^{-2} s^{-1} kPa^{-1} (Amcor Flexibles, Gloucester, UK).

2.3. Preparation of L. monocytogenes cultures and inoculation of fresh-cut lettuce

Three *L. monocytogenes* strains (cultured separately at 4 °C for 14 days or 8 °C for 7 days in accordance to the temperatures being used in the test conditions in 10 ml of tryptic soy broth; TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) from the *Listeria* strain collection at Teagasc Food Research Centre (Moorepark, Ireland; strains 959 - vegetable isolate, 1382 – EUR Lm reference strain, 6179 – food processing plant isolate) were used for all tests in order to follow the European Guidelines for challenge tests on ready-to-eat foods (Beaufort et al., 2014). Equal quantities of each strain were mixed after cultivation for inoculation of fresh-cut produce and diluted to 10^{2-3} CFU g^{-1} in phosphate buffered saline (PBS, pH 7.3, Oxoid BR014, Fannin Healthcare). Aliquots of 0.1 mL of *L. monocytogenes* suspension were distributed uniformly over the lettuce contained within each of the packages immediately before antimicrobial treatments were applied (see section 2.4 below).

2.4. Antimicrobial treatments

The effectiveness of the anti-listerial treatments were tested as follows and compared to controls that received 1 ml of PBS instead:

2.4.1. Lactococcus lactis application

L. lactis subsp. *lactis* DSM 20729 (ATCC 11454) was grown in TSB as described for *L. monocytogenes* in section 2.3 at either 4 or 8 °C. The culture was diluted in 20 ml PBS (pH 7.3) to allow inoculation of fresh-cut produce at 10^3 CFU g^{-1}. Aliquots of 1 mL of *L. lactis* suspension were distributed uniformly over the lettuce contained within each of the packages.

2.4.2. Nisin A application

Commercially available Nisin A (2.5% w/w in salt) (Handary SA, Brussels, Belgium) was suspended in 10 ml of distilled water (100 mg kg^{-1}) in Float-A-Lyzer membranes of 1–5 kDa (Spectrum Laboratories, Inc. California, USA) in 2 L of distilled water for 24 h. The dialyzed solution in the tubes were adjusted with distilled water to 100 mg L^{-1} nisin and autoclaved at 121 °C for 15 min. One part of the stock solution was diluted with sterile water to 50 mg L^{-1} nisin. Both, the 50 and 100 mg L^{-1} nisin was diluted in PBS to 25 and 50 mg L^{-1} nisin for application at a final concentration of 2.5 and 5 mg kg^{-1}. Aliquots of 1 mL of the Nisin A suspension were distributed uniformly over the lettuce contained within each of the packages. Control packages received 1 mL of 0.5x PBS instead.

2.4.3. Atmospheric treatments, package sealing and storage conditions

After completion of the inoculation and the antimicrobial treatment, packs were flushed with gas atmospheres: (a) air, (b) 8 kPa CO_2, 4 kPa O_2, 88 kPa N_2 or (c) 15 kPa CO_2, 1 kPa O_2, 84 kPa N_2 modified atmosphere packaging (MAP) and heat sealed using a vacuum packer (Multivac Mobil 3000, Wolfertschwenden, Germany) as described previously (Scollard et al., 2016) into 18 x 10 cm packs (see also Section 2.2). The packs were then incubated for up to 7 d at either 4 or 8 °C. Oxygen concentrations in the packs were determined immediately before destructive sampling with a Systech 6600 (Systech Instruments, Thame, UK) headspace oxygen analyser.

2.5. Enumeration of L. monocytogenes

Bacterial cell counts were carried out throughout storage from three replicate packs on days 0 (day of inoculation) 2, 5 and 7. The lettuce samples from each package were homogenised for 120 s at 260 pedal strokes per minute in PBS in a 2-fold dilution (20 ml for 10 *g* of lettuce)