Environmental and Experimental Biology (2020) 18: 299–303
http://doi.org/10.22364/eeb.18.29

Brief Communication

Growth potential of *Listeria monocytogenes* in cold smoked sausages with and without fermentation

ISSN 2255-9582

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Abstract

Listeria monocytogenes is a well-known psychrotrophic food pathogen. The aim of the study was to evaluate the growth potential of *L. monocytogenes* in two ready-to-eat cold smoked sausages with and without starter cultures. The goal was achieved using the microbiological challenge test. Sausage samples were artificially contaminated with *L. monocytogenes* and stored at 4 ± 2 °C for 30 days. No increase in *L. monocytogenes* was observed and its concentration in the fermented sausages "Cigarellas" decreased by approximately log 0.1 per day. Significant growth of listeria was observed by day 21 in non-fermented sausages "Barona". In the following days the amount of listeria gradually decreased, and after 28 days it did not differ from the initial amount. The growth potential of *L. monocytogenes* in "Cigarellas" and "Barona" was zero and 0.4, respectively. The products tested were unable to support the growth of listeria. Thus, they can be classified as "Ready-to-eat food unable to support the growth of *Listeria monocytogenes*". Despite the low growth potential, it is desirable to expand the fermentation of uncooked ready-to-eat sausages to achieve zero tolerance for *L. monocytogenes*.

Key words: cold smoked sausages, fermentation, growth potential, *Listeria monocytogenes*.
Abbreviations: a_w, water activity; CFU, colony-forming units; RTE, ready-to-eat.

Introduction

Ready-to-eat (RTE) foods, including cold smoked sausages, are ready for consumption without further preparation, such as thermal processing, and are therefore a major cause of food-borne illnesses. *Listeria monocytogenes* is a major concern in RTE meat products.

L. monocytogenes is a ubiquitous, well-known, non-sporulating, saprophytic bacterium, facultative anaerobe, well adapted to different environmental conditions and is a facultative intracellular pathogen of human and animals. *L. monocytogenes* is classified as a psychrotrophic capable of growth from 1 to 45 °C, including at refrigeration temperatures (Seeliger, Jones 1986). The bacterium is able to grow at a pH of 4.1 to 9.6 (reviewed in Lungu et al. 2009), water activity $a_w \geq 0.90$ (Nolan et al. 1992) and concentration of NaCl ≤ 25.5% (Bergholz et al. 2010). Its ubiquitous nature together with its physiological properties make *L. monocytogenes* a significant food-borne pathogen of RTE products. Listeria are sensitive to heating and die during heat treatment of products (reviewed in Zhu et al. 2005).

Ninety-nine percent of all human cases of listeriosis are food-borne (reviewed in Orsi et al. 2011). Systemic listeriosis mainly occurs in particular risk groups: pregnant women, the elderly, immunocompromised people, and neonates. The gastroenteric form occurs in non-immunocompromised individuals. The EFSA Panel on Biological Hazards (2018) has recognised that more than 90% of invasive listeriosis cases are caused by ingestion of RTE food containing > 2000 colony forming units (CFU) g^{-1}, and that one-third of cases are due to growth in the consumer phase. RTE products like meat and meat products, fish and fish products, and milk and milk products are associated with human listeriosis. A total of 37 food-borne outbreaks caused by *L. monocytogenes* were reported in the EU/EEA with 525 human cases, 182 hospitalisations and 37 deaths during the period of 2008 to 2015. Meat and meat products were responsible for 11 of these outbreaks, causing 126 cases (EFSA Panel on Biological Hazards 2018).

According to European Union regulations, the concentration of *L. monocytogenes* must not exceed 100 CFU g^{-1} of RTE foods. Products with pH ≤ 4.4 or $a_w \leq 0.92$, products with pH ≤ 5.0 and $a_w \leq 0.94$ are automatically considered to belong to the category of RTE foods that do not support the growth of *L. monocytogenes*. Other

categories of products may also belong to this category if scientifically justified (EC 2005). For RTE foods with an extended shelf-life under refrigeration, growth inhibition of *L. monocytogenes* is based upon hurdle technology (formulation of the food, microbial ecology and storage conditions) (Uyttendaele et al. 2007). Listeria are often found in food processing plants. Resin or plastic surfaces contaminated with organic residues with a neutral pH, low temperature and high humidity pose the greatest risk (Chasseignaux et al. 2002). Contamination is possible both throughout the product and from the surface.

Sausages may be preserved by curing, drying, or smoking. Nitrites and nitrates have been widely used as curing agents. Meat sausages can be slowly cured through spontaneous fermentation by microbiota present in the raw materials or introduced during manufacturing (Semedo-Lemsaddek et al. 2016). Fermented sausages are made using protective cultures, i.e. lactic acid bacteria alone or in combination with nitrate reducing catalase positive cocci (Ordóñez, de la Hoz 2007). Antilisterial strains of *Lactiplantibacillus plantarum* (previous name *Lactobacillus plantarum* (Tolvanen et al. 2008) and *Latilactobacillus sakei* (previous name *Lactobacillus sakei*) have been isolated (Ammor et al. 2005) and used as protective starter cultures for meat and meat products (Bredholt et al. 2001). *L. sakei* is known for its ability to produce bacteriocins, and the most studied of these are sakacins (Sawa et al. 2013).

To assess the risk associated with RTE products, it is important to determine the growth potential of *L. monocytogenes* on the food under storage conditions. In accordance with the EURL *Lm* Guidance (2018), the "growth potential (δ) is defined as the difference between the log10 CFU g^{-1} at the end of the challenge test and the log10 CFU g^{-1} at the beginning of the test". In accordance with ISO 20976-1:2019, the "growth potential (Δ) is the difference between the decimal logarithm of the highest concentration of the target microbial population (log_{max}) and the decimal logarithm of the initial concentration of this microbial population (log_i)".

The aim of the work was to determine the growth potential of *L. monocytogenes* in two types of cold smoked sausages, with and without protective cultures, during a period of up to 30 days after production, storing the products at a temperature of 2 to 6 °C. The microbiological challenge test with surface-inoculation of sausages was used.

Materials and methods

Production process

Cold smoked sausages were prepared at the "Ādažu desu darbnīca" Ltd. production plant (Latvia). Production involved several operations. After slicing and mincing, complex additives were added to the meat and stuffed in casings. Heat treatment was performed in smoking chambers, where the sausages were smoked at a temperature of 20 to 22 °C. The humidity was initially set to 90%, after which the program itself gradually reduced the humidity level. The duration of the cycle was 5 days. The product was then transferred to a ripening climate chamber with a relative humidity of 76% and a temperature of 16 to 18 °C. The product was ripened for 3 to 4 weeks until the pH reached the level prescribed for each type of sausage.

According to the original recipes, two types of cold smoked sausages were prepared, i.e. "Barona" and "Cigarellas". For both sausages, a_w was 0.95. The shelf-life specified by the manufacturer was 30 days when stored at 2 to 4 °C.

The formulation of "Barona" (kg per 100 kg) was: chilled beef 70.0, chilled pork 25.0, nitrite salt (Suprasel, Akzo Nobel Salt A/S; sodium chloride 99.4%, sodium nitrite 0.5%) 2.4, Kobi Salami Milano Komple (GBB Group Ltd.; dextrose 35% et al.) 1.2, GDL (D-glucono-1,5-lactone, E575) 0.9; and traditional shirred collagen casing FG 45 RTU (Fabios S.A.) 0.7 m per kilogram of filling. The final product was cylindrical, about 43 mm diameter. The pH level was 5.7 before processing and 4.9 after processing.

The formulation of "Cigarellas" (kg per 100 kg) was: chilled pork 68.4, chilled beef 28.2, nitrite salt (Suprasel, Akzo Nobel Salt A/S, Denmark; sodium chloride 99.4%, sodium nitrite 0.5%) 2.6, Chilli Salami Stiks OG (GBB Group Ltd.; dextrose 30% et al.) 1.5, Aroma Juicy Chicken (GBB Group Ltd.; dextrose 40% et al.) 0.5, Bitec®Starter B2 (Frutarom Savory Solutions GmbH, Austria) 0.8; and edible bovine collagen casing STIX-D Smoke 16/17 (Devro s.r.o.) 3.5 m per kilogram of filling. The final product was cylindrical, about 14 mm diameter. The pH level was 5.9 before processing and 5.4 after processing.

Microbiological testing

The products from three production batches were used for the analysis and 60 g units of the product were tested. Prior to the addition of the test microorganism, each unit was tested for the initial presence of *L. monocytogenes* in 25 grams by the method ISO 11290-1:2017.

Microbiological challenge tests for *L. monocytogenes* growth studies were performed according to ISO 20976-1:2019. The *L. monocytogenes* reference culture ATCC 19112 (Microbiologics™) was used for the challenge test. The inoculation method chosen mimicked the external contamination of the sausage surface. The samples were infected by immersing them in *L. monocytogenes* suspension in peptone physiological saline solution (peptone 1 g L^{-1}, NaCl 8.5 g L^{-1}) for 30 min at 20 °C. The calculated concentration of listeria was 100 CFU mL^{-1}. After infection, the zero day analysis was performed and the remaining samples were stored in sterile containers in a refrigerator at 2 to 6 °C between runs. Samples were periodically tested for *L. monocytogenes* per gram of product by the ISO 11290-2:2017 method. The lower detection limit was 10 CFU g^{-1}.

Statistical analysis

The mean and standard deviation were calculated. A student *t*-test was used to check for differences in bacterial CFU g^{-1} at different product storage times. The significance level was set at $p \leq 0.05$.

Results

Initially, on day zero, no *L. monocytogenes* were found in 25 grams of either of the cold smoked sausages. After artificial inoculation with *L. monocytogenes*, significant growth of listeria was observed by day 14 and day 21 ($p = 0.004$) in sausages "Barona" (Fig. 1). The maximum concentration was reached by day 14, it was 2.6 CFU g^{-1} or 0.4 log higher than the initial concentration. In the following days, the amount of bacteria gradually decreased, and after 28 days it did not differ significantly ($p > 0.05$) from the initial amount. The mean rate of decrease was approximately the same as the mean rate of increase, i.e. 0.3 log per day. According to EURL *Lm* Guidance (2018) the growth potential of *L. monocytogenes* was zero, but according to ISO 20976-1:2019 it was 0.4.

A significant decrease, i.e. a reduction by 1.7 CFU (0.2 log) and 5.0 CFU (0.7 log) in the concentration of inoculated listeria in "Cigarellas" was observed after 8 days ($p = 0.015$) and 12 days ($p = 0.002$) of storage, respectively. No increase in *L. monocytogenes* concentration was observed, but it decreased in the product by approximately 1.3 CFU (log 0.1) per day. After 24 days of storage, it was below the detection limit of the method, i.e. 10 CFU g^{-1} (Fig. 2). The challenge test suggests that "Cigarellas" possessed bactericidal effect to listeria, as the amount of bacteria decreased by 250 CFU (2.4 log) g^{-1}.

The growth potential is considered negligible if the concentration of the target microorganism does not increase by more than 3.5 or 0.5 log CFU g^{-1} from the initial concentration during the test period (ISO 20976-1:2019). In this study, the growth potential of *L. monocytogenes* in "Cigarellas" and "Barona" sausages was zero and 0.4, respectively.

Discussion

Meat processors need to be sure that their products do not support the growth of *L. monocytogenes*, so challenge studies must be performed. For artificial inoculation of cold smoked sausages, the method of simulated surface contamination was chosen. Although the presence of listeria in processing plants has been identified as the most common cause of post-processing contamination of RTE foods (Malley et al. 2015), such contamination can occur at both the post-processing and retail/home level. Sausage casings, natural or artificial, allow listeria to grow (Rivas et al. 2018).

Naturally fermented sausages can be prepared without the use of microbial starters, but with addition of sugar, NaCl and additives such as nitrates or nitrites and spices (Comi et al. 2005; Comi et al. 2020) to control pathogenic bacteria. *Lactobacillus, Staphylococcus* and *Kocuria* spp. are the main representatives isolated from spontaneously fermented sausages, and the species *L. sakei* is most common (Hugas et al. 1993). *L. sakei* uses sucrose (Hugas et al. 1993) and has proteolytic activity, so it is able to hydrolyse meat proteins (Fadda et al. 1999). However, sugar (0.5 to 1.0 %) plays an important role in the acidification process (Gonzalez-Fernandez et al. 2006). In the case of the studied "Cigarellas"sausages, their components "Aroma Juicy Chicken" and "Chilli Salami Stiks" contained dextrose, and calculations show 0.95% content of dextrose in sausage batter, which is sufficient to allow lactobacilli to grow. "Cigarellas" sausages did not promote the growth of listeria and were listericidal (Fig. 2). This can be explained by the addition of a starter. The starter with protective properties against *L. monocytogenes* "Bitec® Starter B2" contained *Lactobacillus sakei, Staphylococcus carnosus* and *Kocuria salsicia*. Commercial starters promote fermentation and increase the safety of fermented meat products by means of acidification of the product and production of bacteriocins (reviewed in Laranjo et al. 2019). Fermented sausages rarely cause outbreaks of listeriosis (European Food Safety Authority 2014).

The "Barona" sausages contained the food additive

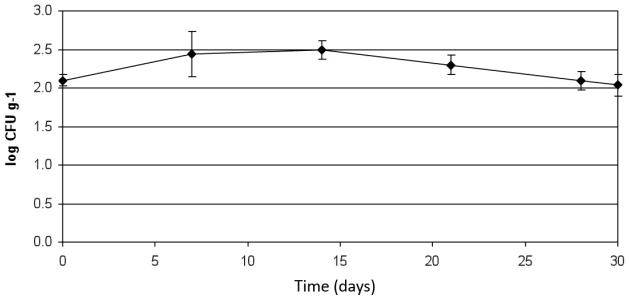

Fig. 1. Changes in *L. monocytogenes* concentration in the sausages "Barona" (log CFU g^{-1}) within 30 days of storage at 2 to 6 °C. Data are means with standard errors.

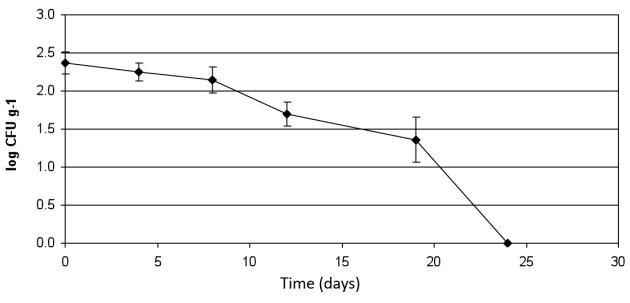

Fig. 2. Changes in *L. monocytogenes* concentration in the sausages "Cigarellas" (log CFU g^{-1}) within 24 days of storage at 2 to 6 °C. Data are means with standard errors.

D-glucono-1,5-lactone, which gives meat products an acidic flavour and becomes a pH-reducing agent after transformation into gluconic acid (Barmpalia et al. 2005). The presence of nitrite and D-glucono-1,5-lactone together with 0.42% dextrose from "Kobi Salami Milano Komple" may explain the insignificant growth potential (0.4) of *L. monocytogenes* in "Barona" sausages and onset of decline of number of listeria after two weeks at a rate of 0.3 log per day (Fig. 1). In two studies without artificial inoculation, Silins and Liepins showed that the number of *L. monocytogenes* decreased by approximately log 0.44 per day (Silins, Liepins 2010) and log 0.27 per day (Silins, Liepins 2011) during the maturation of cold smoked sausages, which was correlated with a decrease in water activity and pH. It is also possible that growth of the natural competitive microorganisms could significantly reduce the counts of *L. monocytogenes* in cases of contamination (reviewed in Thévenot et al. 2006).

The products tested were unable to support the growth of *L. monocytogenes* and can be classified in the category 1.3 of the Commission Regulation (EC) No 2073/2005 (2005) as "Ready-to-eat food unable to support the growth of *Listeria monocytogenes*". Despite the low growth potential, which allows "Barona" sausages to be considered non-growth-promoting for *L. monocytogenes*, it is desirable to expand the fermentation of uncooked sausages or to introduce other anti-listerial measures to achieve zero tolerance for *L. monocytogenes* in RTE meat products.

Acknowledgements

This work was supported by the European Regional Development Fund project "Ādažu desu darbnīca" Ltd. cold smoked sausage and cold smoked beef production technology development, number 1.2.1.1/18/A/002. The authors affirm that this manuscript is an honest, accurate, and transparent account of the study. J.S. and A.S. are employees of the production company "Ādažu desu darbnīca".

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Received 5 December 2020; received in revised form 18 December 2020; accepted 20 December 2020