Antimicrobial activity evaluation of pure compounds obtained from *Pseudoalteromonas haloplanktis* against *Listeria monocytogenes*: Preliminary results

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Abstract

L. monocytogenes is a foodborne pathogen responsible for a serious disease with a high mortality rate, particularly in vulnerable consumers. Recently, the scientific community has shown increasing attention to the search for new natural molecules with antimicrobial activity, aimed at preventing the spread of foodborne diseases. Extremophilic microorganisms, typical of extreme temperature environments, are a valuable source of these molecules. The present work aimed to study the antibacterial activity of four pure compounds derived from a molecule, the pentadecanal, produced by the Antarctic bacterium *Pseudoalteromonas haloplanktis*, against two different pathotypes of *L. monocytogenes*. Growth assays were performed in 96-well polystyrene plates with serial dilutions of the tested compounds at different concentrations (0.6, 0.3, 0.15, 0.07 mg/mL). The plates were incubated at 37°C for 24 h, with a spectrophotometric reading at OD 600 nm. Preliminary results of this study showed that pentadecanal inhibits the growth of *L. monocytogenes*, with a MIC (Minimum Inhibitory Concentration) of 0.6 mg/mL. Acetal, carboxylic acid, and ester did not demonstrate antibacterial activity at the concentrations tested. These findings suggest the possibility of using pentadecanal as a natural antibacterial to improve safety standards along the food supply chain.

Introduction

The foodborne pathogen *L. monocytogenes* is responsible for an invasive infection with a significant morbidity and mortality rate, especially in high-risk groups such as pregnant women, neonates, and immunocompromised individuals. In recent years, the high case fatality rate (13%) has made listeriosis one of the most serious food-borne disease under EU surveillance, after campylobacteriosis and salmonellosis (Pepe *et al.*, 2009; EFSA & ECDC, 2021). In Europe, 2621 cases of invasive listeriosis and 300 related deaths were reported per year, especially associated to the ingestion of raw and RTE (ready-to-eat) foods (meat, milk, and fishery products) (EFSA 2018; EFSA & ECDC, 2021). The predominant serovar isolated from food samples is 1/2a, even though serovar 4b is known to be the cause of the largest number of human listeriosis outbreaks (Jamali *et al*., 2015; Pieta *et al.*, 2018). *L. monocytogenes* can survive a wide range of environmental conditions and can form a biofilm, developing resistance to sanitisers and antimicrobial agents and therefore causing serious problems to food industries (Liu *et al*., 2012; Liu *et al*., 2013; Pacheco *et al*., 2020).

Recently, the scientific community has shown increasing attention to the search for new natural molecules with antimicrobial activity, aimed at preventing the spread of foodborne diseases (Ambrosio *et al.*, 2020; Ceruso *et al.*, 2020; Ceruso *et al*., 2021; Festa *et al.,* 2021). Natural compounds could have plant, animal, or bacterial origin, and assist and/or replace the chemical preservatives used in the food industry.

Extremophilic microorganisms, typical of extreme temperature environments, are a valuable source of these molecules. In fact, they can establish competitive systems, based on the production of active biological substances, capable of counteracting the growth of other microorganisms. Several current research showed that the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 produces a long-chain fatty aldehyde, the pentadecanal (Casillo *et al*., 2017; Ricciardelli *et al*., 2020), endowed with an interesting antibacterial activity against *S. epidermidis* (Papa *et al*., 2013; Parrilli *et al.*, 2015). In this study, we focused on the antibacterial ability of pentadecanal compounds against *L. monocytogenes* to provide more evidence on their potential use as a natural and efficient preservative in the food industry. In addition, some pentadecanal derivatives were obtained (Ricciardelli *et al*., 2020) by modifying its functional group, the corresponding acid, acetal and ester. Derived molecules were also tested for *in vitro* antibacterial activity against two different pathotypes of *L. monocytogenes*.

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Materials and Methods

Pentadecanal and derivate compounds

Four pure molecules were tested to evaluate their antibacterial activity: pentadecanal, pentadecanoic acid (carboxylic acid), pentadecanoic acid methyl ester (ester), and 1,1-dimethoxypentadecane (acetal). The molecules were synthesized from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 at the Department of Chemical Sciences (University of Naples Federico II, Naples, Italy) as described in Ricciardelli *et al.* (2019). The compounds, supplied in

lyophilized form, were resuspended at the Department of Veterinary Medicine and Animal Production using 99.9% pure Dimethyl Sulfoxide (DMSO, Biofroxx, Germany) with a final concentration of 40 mg/mL.

Bacterial strains and culture conditions

The strains used in this work were *L. monocytogenes* H2048 type 4b and H2053 type 1/2a obtained from culture collection centre at the Department of Food Safety, Nutrition and Veterinary Public Health (Istituto Superiore di Sanità, Rome, Italy). The strains were originally isolated from clinical samples and grown in two different culture media: Tryptone soya agar (TSA, Oxoid, United Kingdom) with 0.3% yeast extract and Agar Listeria according to Ottaviani & Agosti (ALOA, Oxoid, United Kingdom). The plates were then incubated at 37°C for 24 hours. For long-term storage, an aliquot was frozen at -20°C in Tryptic Soy Broth (TSB Oxoid, United Kingdom) containing 15% (v/v) glycerol.

Determination of antimicrobial activity and Minimum Inhibitory Concentration

The strains were streaked onto Brain Heart Infusion agar (BHI Oxoid, United Kingdom) plates and incubated at 37°C for 24 hours. A typical colony was selected, inoculated into 5 mL of BHI broth and incubated at 37°C under continuous agitation (200 rpm) for 24 hours. The overnight cultures were normalized to the same optical density (OD_{600nm}) achieving the concentration of 1.5×10^6 CFU/mL (according to the 0.5 McFarland standard turbidity). From the overnight culture obtained from each strain, a 1:1000 dilution in BHI broth was made. The assay was performed in sterile 96-well polystyrene plates (Falcon) with serial dilutions of the compounds tested at different concentrations (0.6, 0.3, 0.15, 0.07 mg/mL) in a final volume of 200 µl. The wells of the first column were inoculated with 190 µl of BHI broth and 10 µl of DMSO (negative control), whereas 190 µl of diluted *L. monocytogenes* culture and 10 µl of DMSO (positive control) were inoculated into the wells of the last column. The central wells received 10 µl of the pure compound with two-fold serial dilutions and 190 µl of diluted culture. The plates were incubated at 37°C for 24 hours and read every two hours using a Glomax multi + detection system (Promega, Madison, USA) microplate reader at OD_{600nm}. Moreover, 100 µl of each suspension was spread on BHI agar and incubated at 37°C for 24 hours. The minimum inhibitory concentration (MIC) value was the lowest concentration of the antimicrobial compound capable of completely inhibiting bacterial growth. At least three experiments were conducted for each antimicrobial compound.

Results

Results showed that pentadecanal is the only molecule capable of inhibiting the growth of *L. monocytogenes*, with a MIC value of 0.6 mg/mL. Acetal, carboxylic acid, and ester showed no antibacterial activity at the concentrations considered in this study. Based on the results, the pentadecanal inhibits the growth of both bacterial strains (Figure 1). In particular, type 1/2a was more sensitive to pentadecanal molecule than type 4b.

Discussion

The interest in alternative antibacterial molecules has been increasing during the last years, supported by research indicating that bioactive molecules produced by some microorganisms have properties that may not only control the growth but also inactivate pathogens in food. The marine environment is rich in biological diversity and can be considered an underexplored source of bioactive molecules. Especially in extreme environmental conditions, such as in Antarctica, it has been reported that cold-adapted bacteria can produce a wide range of compounds (Ricciardelli *et al*., 2018), some of which were found to have a specific action against biofilm formation (Casillo *et al*., 2017). It was demonstrated that the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 produces a long-chain fatty aldehyde, the pentadecanal, that did not show bacterial growth inhibition at tested concentrations but evidenced to counteract *Staphylococcus epidermidis*' biofilm formation (Ricciardelli *et al*., 2020). Our preliminary research intended to explore the potential of pentadecanal and its derivates in inhibiting the growth of *L. monocytogenes*. In fact, neither this molecule nor its derivatives, to our knowledge, were tested before for the evaluation of antilisterial activity.

Results indicate that pentadecanal compound is very effective and possesses antilisteric properties. It was found to be more active against 1/2a strain whereas 4b strain resulted in higher resistance. Compared to the positive controls which achieved concentrations of about 9 Log (CFU/mL) at 24 hours, the growth curves of pentadecanal (Figure 1) do not show an obvious log phase, indicating that the growth of *L. monocytogenes* is inhibited. The strains' behaviour was slightly different. After 24 hours, 1/2a strain reached a

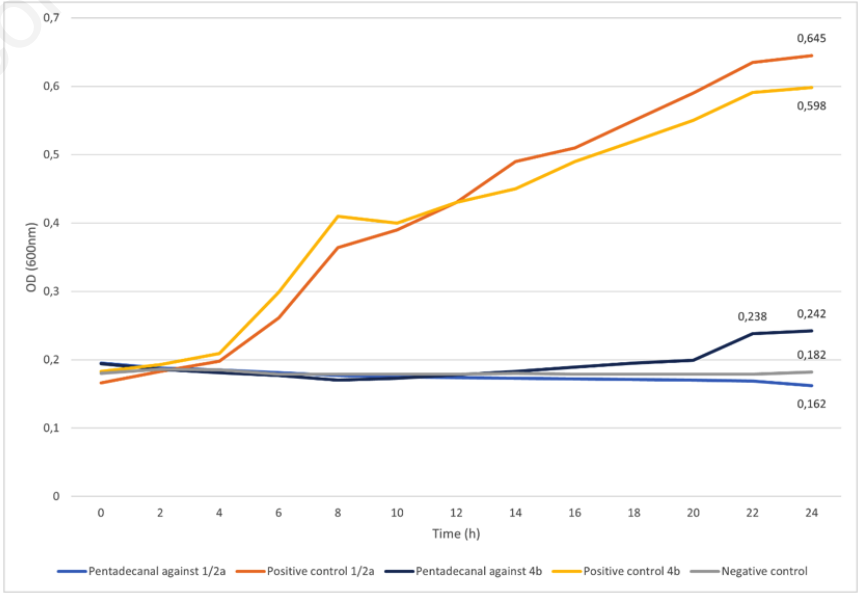

Figure 1. Antibacterial activity of pentadecanal at 0.6 mg/mL concentration against L. *monocytogenes* type 1/2a and type 4b. OD_{600nm} values were measured during 24 hours at different intervals. *L. monocytogenes* strains with DMSO and without compound were used as positive control.

cell density of 1,02 Log (CFU/mL) (OD_{600nm} = 0.162), whereas 4b strain showed a final concentration of 2,19 Log (CFU/mL) (OD_{600nm} = 0.242). The observed strain-dependent antimicrobial susceptibility of *L. monocytogenes* could be due to several factors, such as a difference in microbial membrane composition (Borucki *et al*., 2003; Yuan *et al*., 2017), highlighted the importance of including different strains during the antimicrobial study. Therefore, the assessed antimicrobial activity will be further evaluated on a larger number of strains, including a reference strain.

Pentadecanal derivates (carboxylic acid, acetal, and ester) showed no antibacterial activity. The different response compared to pentadecanal molecule is probably correlated to the difference in their molecular structures. In fact, aldehydes are intrinsically very reactive compounds and readily react with biologically important nucleophile groups of the bacterial cell membrane (Bisignano *et al*., 2001).

The findings of the present work suggest that pentadecanal can represent a potential antimicrobial in controlling *L. monocytogenes* growth. The effective application of this molecule in the food industry could be achieved by using it as food preservatives, active packaging material or natural sanitisers for the food processing environment. It is important to highlight that these preliminary results address the behavior of *L. monocytogenes* in vitro and it could be different in naturally contaminated food.

The mechanisms that explain the pentadecanal anti-listeria activity are not yet well understood, so extensive studies are necessary to validate its efficacy and safety for a proper risk analysis, as required by EU legislation concerning the addition of molecule with antibacterial activity in foods or food contact materials. In previous studies (Ricciardelli *et al*., 2020) biocompatibility with epithelial cells was assessed, but no reports on the effect of pentadecanal as food preservatives could be found in the literature. Toxicity evaluation (cytotoxicity, mutagenicity, and genotoxicity) is encouraged to assess the safe dose and additional studies about the antibacterial activity of these molecules on other foodborne pathogens are underway.

Conclusions

The preliminary results showed a marked antimicrobial activity of the pentadecanal compared to its derivates, highlighting the possibility of using this molecule as an alternative antibacterial. To assess its effectiveness against *Listeria monocytogenes*, additional studies on the anti-biofilm properties are needed. Further investigations are underway to identify potential applications of new compounds as natural food preservatives or in the formulation of innovative food packaging, reducing the risk of contamination by *L. monocytogenes* in the food industry.

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