Thymus vulgaris L. as a possible effective substitute for nitrates in meat products

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Abstract

Nitrates are chemicals found naturally in some foods such as fruit and vegetables or added to others, especially meats, as a preservative. Their use as additives is regulated by European Commission to avoid any risk for human health. In order to reduce or replace the use of these compounds, we investigated the bacteriostatic/bactericidal activity of the essential oil of *Thymus vulgaris* L. against *Listeria innocua*, a non-pathogenic microorganism with the same morpho-cultural traits of *L. monocytogenes*. The study was carried out *in vitro* and *in vivo* on processed meat products, *i.e.* mature salami, by using thyme essential oil. Although the results are preliminary, the antilisterial activity of the thyme essential oil was shown to be similar to that of nitrates.

Introduction

Nitrates are chemical compounds that occur naturally in the environment and are important plant nutrients. Their food sources include fruit, vegetables, drinking water, cheese and meat. They are used as a preservative or to improve the color of processed meat products in which cannot exceed the maximum levels set by the Regulation (EC) No 1333/2008 and subsequent amendments, Regulation (EC) No 1129/2011. Some of the dietary nitrates introduced are converted by oral cavity bacteria into nitrites. After absorption, the latter, in turn, can oxidize hemoglobin reducing the ability of red blood cells to bind and transport oxygen. They may also contribute to the formation of nitrosamines, some of which are carcinogenic (EFSA, 2017). In recent years, due to the risk of using synthetic preservatives, various promising alternatives have been proposed. Among them, the essential oils have received increasing attention as natural additives for their antibacterial, antifungal, antioxidant and aromatizing properties. The use of essential oils in the food area is possible thanks to the GRAS (Generally Recognized As Safe) recognition (FDA 2017). New forms of application to avoid the problems due to low water solubility, high volatility, and strong aroma that can alter the sensorial characteristics of the products are currently under study (Fernández-López *et al*., 2018).

The essential oils are complex mixtures of volatile compounds particularly abundant in aromatic plants, mainly composed of terpenes arising from the mevalonate pathway. These molecules include monoterpenes (hydrocarbon and oxygenated derivatives) and sesquiterpenes (hydrocarbon and oxygenated derivatives). Furthermore, they contain phenolic compounds, which arise from the shikimate pathway (Dhifi *et al*., 2016). The chemical composition of each essential oil varies according to many factors such as plant genotype, altitude, soil, light exposure, climate and time of harvesting (El-Alam *et al*., 2018; Barfroshan *et al.,* 2018 and Boveiri Dehsheikh *et al*., 2019).

Thyme has a long tradition of use in many countries of Europe and worldwide. Its properties have been extensively exploited in traditional medicine for many centuries. It was used as astringent, anthelmintic, carminative, tonic, antiseptic and antimicrobial (Al-Asmari *et al*., 2017). Thyme, in form of essential oil, hydroglyceric extract or poultice, was exploited for bacteriostatic and bactericidal activity to cure skin lesions and excoriations. Currently, it is used in cosmetic preparations useful in the adjuvant treatment of specific dermatological therapies, but also in perfumery, pharmaceutical and food industries as additive (Al-Asmari *et al*., 2017).

Aim of the present work was to assay the potential of *T. vulgaris* as a preservative in salami, evaluating the *in vitro*/*in vivo* bacteriostatic/bactericidal activity of the essential oil against *L. innocua,* a non-pathogenic microorganism with the same morpho-cultural traits of *L. monocytogenes*.

Materials and Methods

T. vulgaris essential oil was purchased at a local herbal store (100% purity).

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In vitro test

The antibacterial activity was determined as previously reported with slight modifications (Vitalini *et al.*, 2016). *Listeria innocua* strain obtained from Microbank ™ Freezer Storage Box of the VESPA Department stock bank was diluted to the final concentration of 10^4 CFU/g (0.5 McFarland turbidity equal to about 1-2x10^8 UFC/mL) and incubated at 37°C for 48 h. It was streaked onto plates of ALOA (Agar Listeria Acc. To Ottaviani and Agosti) medium, and then 20 µL of essential oil were added. Dimethyl sulfoxide (DMSO) was used as solvent to dissolve the essential oil. The plates were incubated at 37°C for 48 h. *L. innocua* at the same conditions, without essential oil, was used as control.

In vivo test

The essential oil at 0.05% was added to the salami mixture. This concertation was selected based on a preliminary study on inoculated sliced salami. *L. innocua* was used at a final concentration of 10^4 CFU/g. A preliminary test showed that 0.025% essential oil was not effective. Five different salami mixtures (A-E) were prepared at the Salvaneschi salami factory, as shown in Table 1. As indicated in the basic recipe of the Salvaneschi factory (Vecchio Varzi D.O.P.), all the mixtures had wine, salt and black pepper grains (22 g of NaCl, 150 mg of potassium nitrate, 200 mL of red wine and 500 mg of black pepper per kg of pork meat). Essential oil was dissolved in red wine. The salami, weighing about 100 g, were subjected to a drying phase, and stored for ripening at 15° ± 0.5°C and 80 % relative humidity for 20 days. At ripening, pH mean was 5.2 ± 0.02 and A_w mean was 0.88 ± 0.003. Twenty-five salami were produced.

On set times T_0 = day 0 (salami preparation), T_1 = 5th day after preparation (a.p.), T_2 = 10th day a.p., T_3 = 15th day a.p., T_4 = 20th day a.p. (the last day), an aliquot of each salami was collected, homogenized, appropriately diluted and streaked on plates with ALOA medium. Dishes inoculated with *L. innocua* were used as control. All plates were incubated at 37°C for 48 h. The *L. innocua* growth was assessed by quantitative analysis.

Statistical treatment of data

All samples were analyzed in triplicate and results expressed as mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA) and comparison among means was determined according to Tukey's honestly significant difference (HSD) test. Significant differences were accepted at P<0.05 and represented by different letters.

Results

In vitro test

L. innocua growing on selective ALOA medium as bluish colonies (Figure 1A) was completely inhibited by the thyme essential oil (Figure 1B). DMSO did not show any inhibitory activity on the bacterial growth.

In vivo test

The obtained results are shown in Figure 2. Nitrates were more effective than the essential oil in reducing bacterial growth, though the differences, at any time point, were not statistically significant P(>.05). No contamination was reported on sample A and E.

Discussion

As previously introduced, the salami samples were contaminated with a *L. innocua* concentration (10^4 UFC/g) higher than that normally present in salami, but useful to evaluate the microbial inhibition.

The results from the *in vitro* test, showing the thyme essential oil efficacy against *L. innocua* after 48 h of incubation, confirmed previous studies (Burt, 2004). Its inhibitory action is due to compounds such as thymol able to modify the bacterial cell membrane permeability thus causing cell death (Burt, 2004). On the other hand, the inhibition of *L. innocua* in the *in vivo* test was less evident and appraisable only after 5-10 days of maturing. As previously reported, different factors such as pH, a_w and NaCl change during seasoning of the salami selecting its microbial flora and preventing

Table 1. Composition of experimental salami.

Sample	Mixture composition of salami
A	Traditional recipe + nitrates
B	Traditional recipe + nitrates + *L. innocua*
C	Traditional recipe + *L. innocua*, without nitrates
D	Traditional recipe + essential oil (0.05%) + *L. innocua*, without nitrates
E	Traditional recipe + essential oil (0.05%), without nitrates

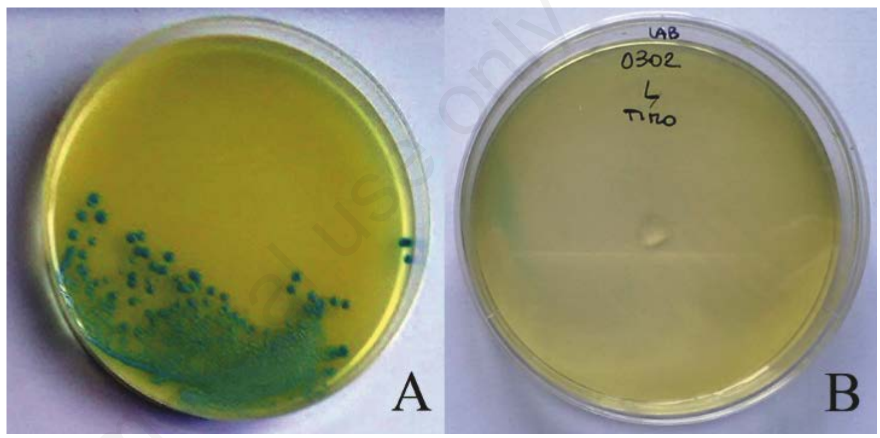

Figure 1. Growth of L. innocua on chromogenic ALOA (Agar Listeria Acc. To Ottaviani and Agosti) medium (A) and its inhibition by thyme essential oil (B).

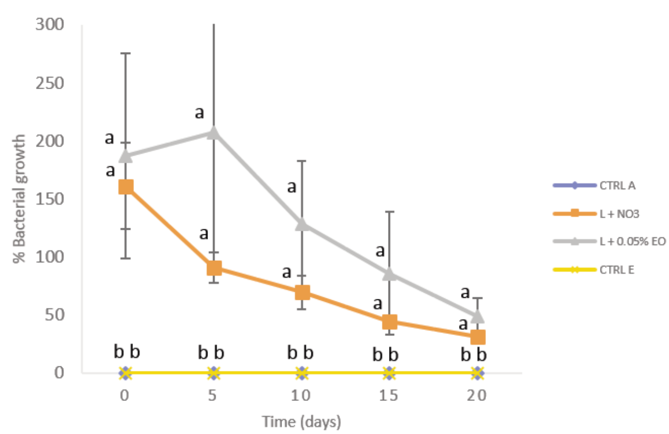

Figure 2. Effects of thyme essential oil (EO) and nitrates on *L. innocua* (L) growth; CTRL A, traditional recipe with nitrates not inoculated; CTRL E; traditional recipe with EO, without nitrates, not inoculated. Results are expressed as percentage of bacterial growth compared with sample C (inoculated with no preservative) set as 100% at each time point. Error bars represent the standard deviation. Significant differences among treatments, at any time point, were accepted at P<0.05 and represented by different letters, according to Tukey's honestly significant difference (HSD) test.

the *Listeria* growth (Meloni, 2015). In addition, the lactic acid bacteria have protective action against various pathogenic microorganisms. For example, they showed an antilisterial activity in sausages after eight weeks of refrigerated storage (Koo *et al*., 2012).

The genus *Thymus* is characterized by chemical polymorphism. Based on *T. vulgaris* essential oil composition, several chemotypes including germacrene D, citral, linalool, (E)- caryophyllene, □-terpinyl acetate, carvacrol, and thymol chemotypes were established (Jarić *et al*., 2015). Nevertheless, European Pharmacopoeia focuses on the thymol chemotype, a mixture of monoterpenes containing 30-70% thymol and 3-15% carvacrol. Therefore, the main constituents are the terpenoid thymol and its phenol isomer carvacrol, which differ only in the position of the hydroxyl group on the phenolic ring (Fachini-Queiroz *et al.*, 2012). Both compounds were evaluated against food-borne pathogens and food spoilage. For example, thymol showed a high inhibitory activity against *Salmonella typhimurium* and *Staphilococcus aureus* by binding to their membrane proteins with hydrogen bonds and altering the cell membrane permeability. It seems that thymol binds the hydrophobic domains of the proteins at lower pH levels (5.5 rather than 6.5) and then spreads in the cell lipid phase (Burt, 2004). Hyldgaard *et al.* (2012) confirmed that thymol interferes with the bacterial membrane, interacting with the membrane proteins, while carvacrol increases the cell membrane permeability to the active ingredients of the essential oil. Other *in vitro* studies have also shown the antibacterial activity of carvacrol as a capacity to reduce the production of diarrheal toxins from *Bacillus cereus* (Burt, 2004). Moreover, García-García (2011) demonstrated that these two natural antimicrobial agents were able to inactivate *L. innocua* in liquid model systems.

Even though this study did not evaluate the sensory properties of salami, the essential oil modified to some extend the aroma and taste traits of treated salami. Another study will further investigate this issue.

Conclusions

In conclusion, to the best of our knowledge, this is the first report on the effects of an essential oil on the growth of *L. innocua* in meat products. Although our findings are preliminary and further studies need to be carried out, we showed that the thyme essential oil exhibited an inhibitory capacity against *L. innocua* in salami similar to that of nitrates. Not least, the addition of essential oil to salami is a new approach that could affect not only the product itself but also its packaging. In fact, in the last years, the active packaging has assumed an important role in the food field (Giarratana *et al*., 2016), where nanoparticles releasing the active antimicrobial compounds according to the food a_w values are also used (Fuenmayor *et al.,* 2013).

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