Evaluation of the antimicrobial activity of thyme essential oil

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Abstract

Enterococci are an important part of the microflora of foods of animal origin. Their effects may be either positive (probiotic action, production of flavour compounds during food ripening) or negative (production of biogenic amines, antibiotic resistance, biofilm formation). The aim of this work was to determine resistance to different concentrations of thyme essential oil and the antibiotic resistance of enterococci isolated from pork (n = 3) and poultry (n = 17). The antibiotic resistance of isolates was determined by the disc diffusion method and the antibacterial effect of thyme essential oil was examined by a microdilution method in 96-well microtitration plates after determination of absorbance at 630 nm (A_{630}). Of the 20 enterococci tested, 85% were resistant to tetracycline, 35% to erythromycin, 15% to ampicillin and 5% to gentamicin. No resistance to vancomycin was detected. All the tested strains of enterococci were able to grow and reproduce at thyme essential oil concentrations of 0.033% and 0.066%. The inhibitory effect of thyme essential oil began at a concentration of 0.099%, though only for 10% of the strains tested. Even the highest tested concentration of thyme essential oil (0.166%) did not inhibit all the tested strains as 25% of enterococcal strains continued to grow. No correlation between antibiotic resistance and resistance to thyme essential oil was detected in the enterococci tested. Thyme essential oil has a potential in the food industry as an inhibitor of spoilage and pathogenic microorganisms, but its antimicrobial activity must be tested in more *in vitro* and *in vivo* experiments and its impact on the sensory properties of foods must also be evaluated.

Antibacterial activity, antibiotics, enterococci, thyme essential oil

Introduction

Vegetable essential oils (EO) are currently widely used in the food industry not merely for their sensory properties, but also for their antioxidant and antimicrobial properties (Burt 2007). Thyme EO is obtained most frequently from species of the genus garden thyme, i.e. *Thymus vulgaris* or *Thymus zygis*, or from a mixture of the two, and mainly contains the isomeric terpenes thymol (30 – 70%) and carvacrol (3 – 15%) (Kresánek 2000). According to Burt (2007), the minimum inhibitory concentration (MIC) of thyme EO is 0.3 $\mu l \cdot ml^{-1}$ for *Listeria monocytogenes*, from 0.45 to 1.25 $\mu l \cdot ml^{-1}$ for *Escherichia coli*, and from 0.2 to 2.5 $\mu l \cdot ml^{-1}$ for *Staphylococcus aureus*. The antibacterial activity of various EO, including thyme EO, has been evaluated *in vitro* by, among others, Soković et al. (2010). They determined the following MIC values for thyme EO: 0.25 $\mu g \cdot ml^{-1}$ for *Bacillus subtilis* and *Staphylococcus aureus* and 0.50 $\mu g \cdot ml^{-1}$ for *Salmonella enteritidis* and *Salmonella typhimurium.* It is extremely important to test the antimicrobial activity of EO not merely in *in vitro* experiments but also when oils are applied directly to foods as individual components as the characteristics of different foods can greatly influence the effects of EO. Selim (2011) monitored the effects of the addition of thyme EO at concentrations of 0.5% to 1% on the growth of vancomycin-resistant enterococci and *Escherichia coli* at 7 °C. On the basis of his results, he recommends the thyme EO be added to food products such as, for example, ground beef. Quattara et al. (2001) evaluated the effects of thyme EO on both antibacterial activity and prolonging shelf life and also the sensory properties of shrimp. While a thyme EO concentration of 0.9% had no negative effects on either the taste or appearance of pre-cooked shrimp, an EO concentration of

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1.8% had negative effects on both. In addition to direct application in foods, other methods of EO use are also being investigated. Emiroglu et al. (2010) applied thyme and oregano EO (0% to 5%) to soya protein-based edible packaging intended for packing semi-finished products made of fresh beef. The antimicrobial action of such packaging was confirmed under *in vitro* conditions even at the lowest concentration of 1%, with the antimicrobial action intensifying with increasing EO concentration.

Enterococci are an important group of microorganisms present in raw materials and foods of animal origin. Traditionally, enterococci are classified as belonging to the group of lactic acid bacteria (Holzapfel and Wood 1995). Due to their presence in the gastrointestinal tract, enterococci are often regarded as an indicator of faecal contamination of the food chain (Giraffa 2002; Folquie Moreno et al. 2006). However, enterococci are also considered part of the natural microflora of food and not just an indicator of poor hygiene (Klein 2003). Similarly to other lactic acid bacteria, some strains of enterococci are used as starter or protective cultures, as feed additives and as probiotics. Enterococci are used in fermentation processes for the production of fermented foods where they contribute to maturation and the development of aroma, probiotic properties and the production of antimicrobial substances (Giraffa 2002; Klein 2003; Folquie Moreno et al. 2006). Meat-isolated enterococci have been found to demonstrate metmyoglobin-reducing activity (Ariharia et al. 1994) and thus help maintain the red colour of fresh meat. In addition to enterococci that represent the original flora in traditional fermented products, enterococci such as *E. faecium* F688 can also be used as commercial probiotic strains in fermented meat products. Enterococcal strains are extremely active in sausages, in which they may reach levels of 10^7 CFU·g^{-1} (Hugas et al. 2003).

In addition to the advantages given above, enterococci are also known to have negative effects. Magnus et al. (1988) found that *Enterococcus* strains such as *E. faecalis* and *E. faecium* are involved in the spoilage of canned meat products such as canned ham. Teuber et al. (1996) found that enterococci are generally resistant to temperature, pH and the presence of salt which means they can multiply, increase in number and consequently cause spoilage of meat products. The decrease in pH and proteolysis during meat fermentation create favourable conditions for the production of biogenic amines by microorganisms (Foulquie Moreno et al. 2006). Enterococci are also characterized by a broad spectrum of resistance to antibiotics (natural / internal and acquired / transferred). Examples of natural resistance are resistance to vancomycin (VanC type) in *E. gallinarum*, resistance to streptogramins in *E. faecalis* and resistance to isoxazolyl penicillins, cephalosporins, monobactams and aminoglycosides (at low levels), lincosamides (most frequent) and polymyxins. Resistance to ampicillin (especially in *E. faecium*), tetracyclines, macrolides, aminoglycosides (high levels), chloramphenicol, trimethoprim / sulfamethoxazole, quinolones and streptogramins (in *E. faecium* and related species) is acquired resistance, similarly to resistance to glycopeptides (vancomycin) (Klare et al. 2001). Enterococci are also known for their ability to exchange genetic information by conjugation. Teuber et al. (1999) reported the successful transfer of antibiotic resistance genes from several species of the genus *Enterococcus* isolated from meat products (sausages, bacon and raw meat) into the collection strain *E. faecalis* JH2-2.

The aim of this study was to investigate the resistance of enterococci isolated from pork and poultry meat to selected antibiotics and resistance to various concentrations of thyme essential oil, and to search for a possible link between these properties.

Materials and Methods

Isolation and identification of enterococci

After 48 ± 2 hours of cultivation of samples at 37 ± 1 °C on Slanetz-Bartley agar (HiMedia, India), typical enterococci colonies (n = 3 from pork and n = 17 from poultry meat) were inoculated onto selective Bile Esculin Azide agar

(Biokar, France) and cultured at 37 ± 1 °C for 24 hrs. Esculin hydrolysis, a catalase-negative test and a positive Pyrrolidonyl-Arylamidase test (Pliva-Lachema, Czech Republic) confirmed the strains as belonging to the genus *Enterococcus*. A commercial En-coccus test (Pliva-Lachema, Czech Republic) was used for species identification.

Determination of antibiotic resistance

The antibiotic resistance of isolates was determined by the disc diffusion method according to CLSI recommendations (2012). The following antibiotic discs (HiMedia, India) were used: ampicillin 10 µg/disc, erythromycin 15 µg/disc, gentamicin 120 µg/disc, tetracycline 30 µg/disc and vancomycin 30 µg/disc.

Antibacterial activity of thyme EO

The antibacterial effects of thyme EO were determined by the microdilution method in a 96-well Microtiter plate. Thyme EO (Hanus, Slovakia) was added in the required amounts to tryptone soya broth (HiMedia, India) at 37 – 40 °C to achieve final concentrations of 0.033%, 0.066%, 0.099%, 0.133% and 0.166%. Ethanol was then added to each sample (final concentration of 0.0165%). In addition to samples with different thyme EO concentrations, negative control samples (with thyme EO and without enterococci) and positive control samples (with enterococci and without thyme EO), as well as control samples to determine the effects of ethanol were tested. A 24-hour culture of selected enterococcal strains was prepared by cultivation on Plate count agar (HiMedia, India) at 37 ± 1 °C. Subsequently, a bacterial culture was prepared in Mueller Hinton broth (HiMedia, India) to be used for experiments. The initial bacterial suspension density was around 6 – 7 log CFU·ml^{-1}. The bacterial suspension (20 µl) was added to 180 µl of thyme EO solution of the appropriate concentration and the Microtiter plate was then cultured for 24 ± 2 hrs at 37 ± 1 °C. The growth of enterococci was determined by absorbance measurement at 630 nm (A_{630}) on a BioTek EL 808 spectrophotometer (BioTek, USA).

Results and Discussion

Antibiotic resistance

The results for the antibiotic resistance of the tested enterococci strains assessed by the disc diffusion method are given in Table 1. Of the 20 enterococci strains tested (pork: n = 3 and poultry: n = 17), 85% were resistant to tetracycline, 35% to erythromycin, 15% to ampicillin and 5% to gentamicin. No resistance to vancomycin was found in the enterococcal strains tested. Kročko et al. (2007) evaluated 75 enterococcal isolates from meat (pork, beef and poultry) and found that 56% of the strains were resistant to tetracycline, 27% to ampicillin, 25% to gentamicin, 15% to vancomycin and 15% to erythromycin. In a study conducted by Koluman et al. (2009), 88% of beef samples and 72% of poultry meat samples were contaminated with enterococci. The authors of the study considered the fact that the strains were resistant to at least two of the tested antibiotics as particularly alarming. Four of the enterococci strains were even evaluated as vancomycin resistant, all four belonging to the *E. faecalis* strain from chicken meat. The results of some studies indicate that *Enterococcus* spp. commonly contaminate retail meats and that the differences in antibiotic resistance among various isolated strains reflect the use of permitted antibiotics in the rearing of different types of livestock for meat production (Hayes et al. 2003).

Antibacterial activity of thyme EO

The antibacterial effects of thyme EO (at different concentrations) on enterococci after 24-hour cultivation at 37 ± 1 °C were determined by absorbance measurement at 630 nm. The absorbance values measured in the experimental samples were then compared with the absorbance values of the positive and negative control samples at the beginning and end of the experiment. At the beginning of the experiment, the ascertained absorbance values of the experimental samples and the positive and negative control samples were comparable. The antibacterial effect of different thyme EO concentrations on the enterococci strains tested is given in Table 2. These results allow to state that the most sensitive of the enterococcal strains tested for the action of thyme EO were *E. faecium* 43 and *E. casseliflavus* 15 isolated from poultry meat. The highest resistance to the effects of thyme EO, on the other hand, was found in the strains *E. faecalis* 66 and *E. faecalis* 3M isolated from poultry meat and *E. faecium* 184, *E. faecium* 282 and *E. mundtii* 296 isolated from pork. The percentages

of enterococcal strains inhibited by test concentrations of thyme EO are shown in Fig. 1. The results indicate that the tested enterococcal strains isolated from poultry and pork meat exhibited different susceptibility to the applied concentrations of thyme EO. The inhibitory effect of thyme EO was first expressed at a concentration of 0.099%, though only for 10% of the strains tested. Not even the highest thyme EO concentration tested (0.166%), however, proved capable of inhibiting all of the strains tested as up to 25% of enterococcal strains continued to grow, with the highest resistance being shown by enterococci isolated from pork. The effects of applied thyme EO concentrations on the sensory properties of foods were not evaluated.

Table 1. Evaluation of the antibiotic resistance of enterococcal strains

Enterococcal strains	Resistance (R) and susceptibility (S) of isolated strains ATB discs					Origin
	VAN 30	TET 30	ERY 15	AMP 10	GEN 120	
E. faecalis 1	S	R	S	S	S	
E. faecalis 2	S	R	S	S	S	
E. faecalis 9	S	R	R	S	S	
E. faecalis 15	S	R	R	S	S	
E. faecalis 18	S	S	S	S	S	
E. faecalis 22	S	R	S	S	S	
E. faecalis 31	S	R	R	S	R	
E. faecalis 40	S	R	S	S	S	
E. faecium 43	S	R	R	S	S	Poultry meat
E. faecalis 50	S	R	R	S	S	
E. faecalis 66	S	R	S	S	S	
E. faecalis 67	S	R	S	S	S	
E. faecalis 3M	S	S	S	S	S	
E. casseliflavus 15P	S	R	S	R	S	
E. gallinarum 35	S	R	R	R	S	
E. faecalis 327	S	R	S	S	S	
E. faecalis 330	S	R	R	S	S	
E. faecium 184	S	R	S	S	S	
E. faecium 282	S	R	S	S	S	Pork meat
E. mundtii 296	S	S	S	R	S	

S – susceptible, R – resistant, ATB – antibiotics (VAN 30 – vancomycin, TET 30 – tetracycline, ERY 15 – erythromycin, AMP 10 – ampicillin, GEN 120 – gentamicin)

Comparison of the results of the EO antibacterial effect with the results produced by other authors is problematic because the designs of individual experiments were different in relation to, for example, the EO composition, the strains of microorganisms tested, the media used and the conditions of cultivation, etc. The results of at least several works in the available scientific literature in which the effects of thyme EO were studied might be mentioned. Hammer et al. (1999) stated that the lowest inhibitory concentration of thyme EO on *Candida albicans* and *Escherichia coli* was 0.03% which is less than was found in those enterococci tests. This may be explained by the well-known observation that enterococci are able to grow in extreme conditions, e.g. in 40% bile, 6.5% NaCl, a pH of 9.6 and temperatures of 10 – 45 °C. Selim (2011) evaluated the antibacterial effects of 11 EO on vancomycin-resistant enterococci.

Table 2. Evaluation of antibacterial activity of thyme EO on enterococcal strains

Enterococcal strains	Concentration of thyme essential oil [%]					Origin
	0.033	0.066	0.099	0.133	0.166	
E. faecalis 1	+	+	+	-	-	
E. faecalis 2	++	++	+	-	-	
E. faecalis 9	+	+	+	-	-	
E. faecalis 15	++	+	+	-	-	
E. faecalis 18	+	+	+	-	-	
E. faecalis 22	+	+	+	+	-	
E. faecalis 31	++	++	+	-	-	
E. faecalis 40	+	+	+	+	-	Poultry meat
E. faecium 43	++	++	-	-	-	
E. faecalis 50	+	+	+	+	-	
E. faecalis 66	+	+	+	+	+	
E. faecalis 67	+	+	+	+	-	
E. faecalis 3M	+	+	+	+	+	
E. casseliflavus 15P	+	+	-	-	-	
E. gallinarum 35	++	++	+	-	-	
E. faecalis 327	+	+	+	+	-	
E. faecalis 330	++	++	+	-	-	
E. faecium 184	+	+	+	+	+	
E. faecium 282	+	+	+	+	+	Pork meat
E. mundtii 296	+	+	+	+	+	

++ moderately stimulating effect of thyme EO on enterococcal growth, + enterococcal growth, - enterococcal inhibition

His results show that the most effective EO on the bacteria tested was thyme oil with MIC_{90} and MBC_{90} for vancomycin-resistant enterococci at 0.25% and 0.5%, respectively. The addition of thyme EO at 0.5% and 1% caused a statistically significant reduction in the rate of growth of vancomycin-resistant enterococci in cheese and meat stored at 7 °C.

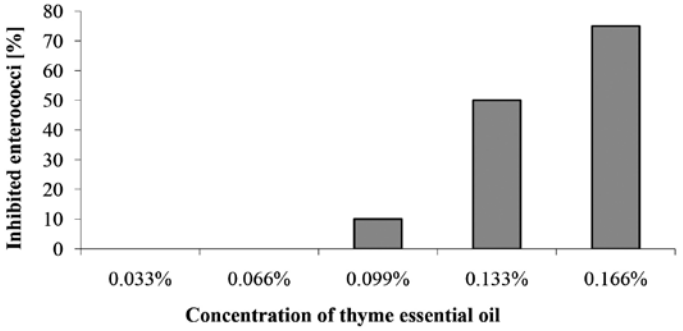

Fig. 1. Effects of various concentrations of thyme EO on enterococcal strains tested after 24-hour aerobic static cultivation at 37 ± 1°C

Sanjuas-Rey et al. (2012) evaluated the effect of various concentrations (0.010%, 0.025% and 0.050%) of thyme and oregano EO on the microbiological quality

of chilled squid (*Loligo vulgaris*). They found thyme and oregano EO to have a significant effect on psychrotrophic representatives of the family *Enterobacteriaceae*. Higher concentrations of both EO tested also reduced lipid oxidation levels.

Any correlation between resistance to antibiotics and resistance to thyme EO in the enterococci tested in this study was not found. However, negative or positive effects of plant extracts on the frequency of spontaneous mutations of microorganisms leading to antibiotic resistance have been reported in some works (Bírošová et al. 2007; Mošovská and Bírošová 2012).

Conclusions

On the basis of the results, it can be concluded that essential oils such as thyme EO have a potential use in the production of foods as a substance improving microbiological quality and safety and prolonging shelf life. In addition to further *in vitro* experiments, it is also necessary to test the antibacterial effects of EO when applied directly to foods, as individual food components may influence EO action on microorganisms and as high EO concentrations may adversely affect the sensory properties of foods. The use of substances with an antimicrobial activity may not always be suitable in the fight against microbial resistance as the mechanisms of mutual influence between input substances (antimutagen-antibiotic or antimutagen-antibiotic-mutagen) remain unclear. It is, therefore, necessary to further investigate these interactions and their impact on the development of resistance.

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