JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ENTEROCOCCI AND THEIR RESISTANCE TO ANTIBIOTICS AND THYME ESSENTIAL OIL

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ARTICLE INFO

Received 10. 10. 2013
Revised 21. 11. 2013
Accepted 16. 12. 2013
Published 1. 2. 2014

Regular article

OPEN ACCESS

ABSTRACT

Enterococci are important part of microflora of food animal origin. They have positive (probiotic effect, production flavor compounds during food ripening) and also negative (production biogenic amine, antibiotic resistance, biofilm production) properties. The aim of this work was to determine resistance to different concentrations of thyme essential oil and antibiotic resistance of enterococci isolated from pork (n=3) and poultry (n=17). The antibiotic resistance of isolates was determined by disc diffusion method and the antibacterial effect of thyme essential oil was assayed by a microdilution method in 96-well microtitration plates after determination of absorbance at 630 nm (A_{630}). Of 20 tested enterococci, 85 % were resistant to tetracycline, 35 % to erythromycin, 15 % to ampicillin and 5 % to gentamicin. No resistance to vancomycin was detected. All tested strains of enterococci were able to grow and reproduce at concentrations of thyme essential oil 0.033 % and 0.066 %. Inhibitory effect of thyme essential oil began at a concentration of 0.099 %, but only for 10 % of the tested strains. Even the highest concentration tested thyme essential oil 0.166 % did not inhibit all the tested strains, because 25 % of enterococcal strains continued to grow. No correlation between antibiotic resistance and resistance to the thyme essential oil was detected for tested enterococci. The thyme essential oil has potential for using in food industry to inhibit spoilage or pathogenic microorganisms, but it is necessary to test antimicrobial activity in other in vitro and in vivo experiments and also in experiments with impact on the sensory properties of food.

Keywords: Antibiotics, thyme essential oil, enterococci, antibacterial activity

INTRODUCTION

Traditionally enterococci are considered as part of the lactic acid bacteria (**Holzapfel and Wood, 1995**). Enterococci have as their main habitat the gastrointestinal tract of humans and warm-blooded animals, but they also occur in soil, surface waters and on plants and vegetables, due to their ability to grow and survive under severe environmental conditions (**Murray, 1990; Giraffa 2002**). Because of their occurrence in gastrointestinal tract, enterococci are often used as indicator of faecal contamination through food production chain (**Giraffa, 2002; Folquie Moreno *et al.*, 2006**). However, enterococci are now also considered as normal parts of the food microflora and not only as indicator for poor hygiene (**Klein, 2003**).

Like most other lactic acid bacteria, some enterococcal strains are used as starter or protection cultures or feed supplements as well as probiotics. Enterococci are applied in fermentation process of fermented food production because their contribution to ripening and aroma development, probiotic properties and the production of antimicrobial substances (**Giraffa, 2002; Klein, 2003; Folquie Moreno *et al.*, 2006**). The breakdown of lactose and citrate during cheese ripening gives rise to a series of volatile compounds, such as acetaldehyde, ethanol, diacetyl, acetone, and acetoin, which may further contribute to flavour. In these regard, many *E. facalis* and *E. faecium* strains isolated from dairy products were shown to be good producer of acetaldehyde, ethanol, diacetyl, and acetoin when grown in milk, thus further contributing in the development of aroma and flavour of cheese (**Andrighetto *et al.*, 2001; Sarantinopoulos *et al.*, 2001**). It seems, therefore, that enterococci have the potential to actively contribute to the flavour development in fermented dairy products (**Giraffa, 2003**).The biochemical activities of enterococci in the sausage matrix have not been studied as in the case of dairy products. They might contribute to sausage aromatisation by their glycolytic, proteolytic and lipolytic activities (**Sarantinopoulos *et al.*, 2001**). Metmyoglobin reduction activity has been described for meat enterococci (**Ariharia *et al.*, 1994**), with a possible role of maintaining the red colour of fresh meat. Enterococci, *E. faecium* and *E. faecalis*, but especially *E. faecium* represent one of the lactic acid bacteria species that can be found in relatively high numbers during meat fermentation. They may contribute, together with lactobacilli, to the fermentation. Except this enterococcal natural flora in traditional artisanal fermented products, enterococci, for example *E. faecium* F688 can be used also as a commercial, probiotic strain in meat fermented products. This strain is also very competitive in sausages reaching 10^7cfu.g^{-1} at the end of the ripening (**Hugas *et al.*, 2003**).

Some enterococci of dairy and also of meat origin have also been reported to produce bacteriocins (enterocins) inhibitory against food spoilage and pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Vibrio cholera, Clostridium* spp., and *Bacillus* spp. (**Giraffa, 2003; Hugas *et al.*, 2003**).

However, enterococci except theses positive properties are also infamous for their negative effects. Research of **Magnus *et al.* (1988)** has shown that enterococcal strains *E. faecalis* and *E. faecium* have been implicated in spoilage of cured meat products, such as canned hams and chub-packed luncheon meats. **Teuber *et al.* (1996)** reported that enterococci are obviously resistant to temperature, pH and salinity effect, they may multiplay to high numbers and cause spoilage of processed meats.

The growth of enterococci of a dairy origin in milk and milk products, leading to formation of significant levels of biogenic amines, has been observed. Also during meat fermentation, the microbial growth, the acidification and the proteolysis offer favourable conditions for biogenic amines production (**Foulquie Moreno *et al.*, 2006**).

Enterococci possess a broad spectrum of antibiotic resistances (natural/intrinsic and acquired/transferable). Examples of intrinsic resistance are vancomycin (VanC type) resistance in *E. gallinarum*, and resistance towards streptogramins in *E. faecalis*, as well as resistance to isoxazolylpenicillins, cephalosporins, monobactams, aminoglycosides (low level), lincosamides (mostly),

andpolymyxins. The resistance to ampicillin (especially in *E. faecium*), tetracyclines, macrolides, aminoglycosides (high level), chloramphenicol, trimethoprim/sulfamethozaxole, quinolones, and streptogramins (in *E. faecium* and related species) are acquired, as well as resistance towards glycopeptides (vancomycin) (**Klare *et al.*, 2001**).

Enterococci are also known for their capacity to exchange genetic information by conjugation. **Teuber *et al.* (1999)** reported successful transfer of antibiotic resistant determinants from several species of *Enterococcus* isolated from meat products (salami, pancetta and raw meat) into the collection strain *E. faecalis* JH2-2.

An important property of enterococci is ability of some strains to form biofilm. Biofilm production can promote increase resistance to antibiotics and other antimicrobials (**Tsikrikonis *et al.*, 2012).** In food industry, biofilm may be a source recalcitrant contamination, causing food spoilage and are possible sources of public health problems such as outbreaks of foodborne pathogens (**Simões *et al.*, 2010**).

Plant essential oils are presently widely used in food industry because their not only aroma substances, but also for their antioxidant and antimicrobial properties against many pathogenic microorganisms as well as microorganisms causing food spoilage (**Burt, 2007**).

The objective of our study was to determine resistance to different concentration of thyme essential oil and antibiotic resistance of enterococci isolated from pork and poultry.

MATERIAL AND METHODS

Isolation and identification of enterococci

After 48 ± 2 h cultivation of samples at 37 ± 1 °C on Slanetz-Bartley agar (HiMedia, India), suspected colonies of enterococci (n=3 from pork and n=17 from poultry) were inoculated on selective bile esculine azide agar (Biokar, France) and cultivated at 37 ± 1 °C for 24 h. Esculine hydrolysis, catalase negative test and pyrrolidonylarylamidase positive test (Pliva-Lachema, Czech republic) indicated the presence of *Enterococcus* genus. A commercial Enterococcus test (Pliva-Lachema, Czech republic) was used to species determination.

Antibiotic susceptibility test

The antibiotic resistance of isolates was determined by disc diffusion method according to the recommendations of **CLSI (2012)**. The following antimicrobial drugs (HiMedia, India) were used: 10 µg ampicillin (AMP), 15 µg erythromycin (ERY), 120 µg gentamicin (GEN), 30 µg tetracycline (TET), and 30 µg vancomycin (VAN).

Antibacterial assay of thyme essential oil

The antibacterial effect of thyme essential oil was assayed by a microdilution method in 96-well microtitration plates. The thyme essential oil (Hanus, Slovakia) was added in the required amount to the tryptic soya broth (HiMedia, India) (temperature 37 – 40°C) to achieve final concentrations 0.033 %, 0.066 %, 0.099 %, 0.133 %, 0.166 %. The ethanol was added to each sample to the concentration 0.0165 %. Except samples with different concentrations of the thyme essential oil, also positive and negative control samples as well as control sample for ethanol effect were tested.

The cultures of enterococci were grown at 37 ± 1 °C on plate count agar (HiMedia, India) for 24 hours. Freshly prepared bacterial cultures in Mueller Hinton Broth (HiMedia, India) were used for experiments. The initial density of bacterial suspension was approximately 6 – 7 log cfu.ml^{-1}. This bacterial suspension (20 µl) was added to 180 µl of tested thyme essential oil solution in microtitrate plate and cultured for 24 ± 2 hours in thermostat at 37 ± 1 °C. The microbial growth was determined by absorbance at 630 nm (A_{630}) using spectrophotometer BioTek EL 808 (BioTek, USA).

RESULTS AND DISCUSSION

Antibiotic susceptibility

Resistance to antibiotics of isolated enterococci performed by disc diffusion method is shown in table 1.

Table 1 Presence of resistant and sensitive strains of enterococci isolated from poultry (n=17) and pork (n=3)

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

Legend: S – susceptibility, R – resistance, ATB – antibiotics (VAN 30 – vancomycin, TET 30 – tetracycline, ERY 15 – erythromycine, AMP10 – ampicillin, GEN120 – gentamicin)

Of 20 tested strains, 85 % were resistant to tetracycline, 35 % to erythromycin, 15 % to ampicillin and 5 % to gentamicin. No resistance to vancomycin was detected. **Kročko *et al. (2007)*** found from 75 isolates of enterococci from meat (pork, beef, poultry) 56 % resistant to tetracycline, 27 % to ampicillin, 25 % to gentamicin, 15 % to vancomycin and also 15 % to erythromycin. In study held by **Koluman *et al. (2009)*,** 88 % of beef samples and 72 % of chicken samples were contaminated with enterococci and the strains of concern were resistant to at least two types of antibiotics. Four strains were identified as vancomycin-resistant enterococci, four of which were *E. faecalis* and originated from chicken. Results of recent studies indicate that *Enterococcus* spp. commonly contaminated retail meats and that dissimilarities in antimicrobial resistance patterns among enterococci recovered from different meat types may reflect the use of approved antimicrobial agents in each food animal production class **(Hayes *et al.*, 2003)**

Antibacterial assay of thyme essential oil

The antimicrobial effect of various concentrations of thyme essential oil against tested enterococci after 24 hours of cultivation at 37 ± 1 ° C was monitored by measuring the absorbance at 630 nm and comparing the measured absorbance values with the positive (sample with enterococci and without thyme essential oil) and of negative (sample without enterococci and thyme essential oil) controls at the beginning and end of the experiment. At the start of the experiment were measured absorbance values in experimental samples and in samples of the positive and negative control comparable.

All tested strains of enterococci were able to grow and reproduce at concentrations of thyme essential oil 0.033 % and 0.066 %, which results from the increase of values A_{630} at these concentrations compared with the A_{630} of positive control at the beginning of the experiment. By comparing the values of A_{630} at concentrations of thyme essential oil 0.033 %, respectively in some cases, at a concentration of 0.066 %, with the values of the positive control A_{630} after 24 hours, may be concluded that in the case of strains of *E. faecalis* 2, *E. faecalis* 15, *E. faecalis* 31, *E. faecium* 43, *E. gallinarum* 35 or *E. faecalis* 330 measured absorbance values were even slightly higher, which would suggest even a slight stimulation effect.

Concentration of thyme essential oil 0.099 % caused that two strains of enterococci - *E. faecium* 43 and *E. casseliflavus* 15, did not change after 24 hours of cultivation values of A_{630}, compared to a positive control at the beginning of the experiment. Because the growth of enterococci was evaluated only through the values of A_{630} and another cultivation method absent, can not be concluded whether the concentration of thyme essential oil 0.099 % had to strains *E. faecium* 43 and *E. casseliflavus* 15 inhibitory or bactericidal effect too.

The tested strains of enterococci *E. faecalis* 22, *E. faecalis* 40, *E. faecalis* 50, *E. faecalis* 67 and *E. faecalis* 327 did not change values of A_{630} after 24 hours of cultivation wit comparison to the positive control at the beginning of the experiment, only at concentrations 0.166 % of thyme essential oil. On the other hand, the strains of enterococci *E. faecalis* 3M, *E. faecalis* 66, *E. faecium* 184, *E. faecium* 282 *and E. mundtii* 296 at the concentration of thyme essential oil 0.166

% increased the A_{630} values after 24 hours of cultivation in comparison with the positive control at the beginning of the experiment, but these values did not reach the values of A_{630} positive control after 24 hours.

On the basis of these results, it can be concluded that strains *E. faecium* 43 *and E. casseliflavus* 15 isolated from poultry were the most sensitive to the thyme essential oil. The highest resistance to the action of thyme essential oil showed strains of *E. faecalis* 66 and *E. faecalis* 3M which have been isolated from poultry and *E. faecium* 184, *E. faecium* 282 and *E. mundtii* 296 which have been isolated from pork.

The percentage of enterococcal strains, which where inhibited at tested concentrations of thyme essential oil are shown in fig. 1

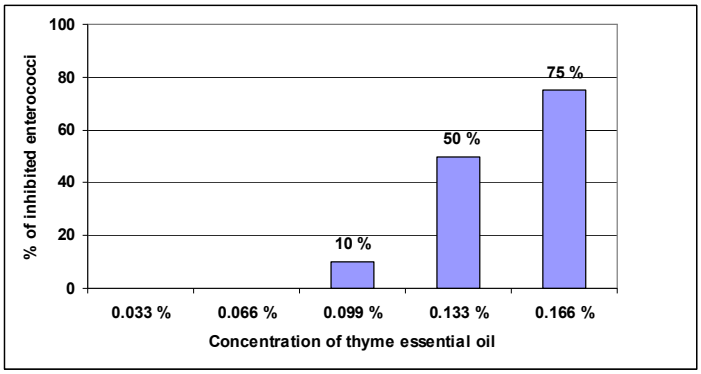

Figure 1 The effect of different concentrations of thyme essential oil on tested strains of enterococci after aerobic static cultivation at 37 ± 1°C after 24 hours

Can be concluded that the tested strains of enterococci isolated from poultry and pork showed different sensitivity to the applied concentrations of thyme essential oil. Inhibitory effect of thyme essential oil began at a concentration of 0.099 %, but only for 10 % of the tested strains. Even the highest concentration tested thyme essential oil 0.166 % did not inhibit all the tested strains, because up to 25 % of enterococcal strains continued to grow.

The comparison of results of essential oil antimicrobial activity with results of other authors is difficult, because the conditions of the experiments are modified. For example, the differences are for compositions of the essential oils, the tested strains of microorganisms, used medium and the cultivation conditions and so on. From the available scientific literature may be mentioned some results with impact to thyme essential oil. **Hammer *et al.* (1999)** reported that thyme essential oil had the lowest inhibitory concentration of 0.03 % against *Candida albicans* and *Escherichia coli*, what is lower than was found in our experiments with tested enterococci. **Selim (2011)** evaluated eleven essential oils for their antibacterial properties against vancomycin resistant enterococci and *E. coli* O157:H7. His results showed that the most active essential oil against tested bacteria was thyme oil with MIC_{90} (minimum inhibitory concentration) and MBC_{90} (minimum bactericidal concentration) for vancomycin resistant enterococci strains of 0.25 % and 0.5 %, respectively. The addition of thyme oil at concentration 0.5 % and 1 % caused best significant reduction in the growth rate of vancomycin resistant enterococci in cheese and meat at 7 °C. **Sanjuas-Rey *et al.* (2012)** evaluated the following concentrations of thyme and oregano essential oils 0.010 %, 0.025 % and 0.050 % on the quality of refrigerated squid (*Loligo vulgaris*). They found a significant effect of thyme and oregano essential oils against representatives of psychrotrophic bacteria from family *Enterobacteriaceae*. Higher concentrations tested both essential oils showed also a lipid oxidation reduction.

No correlation between antibiotic resistance and resistance to the thyme essential oil was detected for tested enterococci. However, in some works (**Bírošová *et al., 2007*; Mošovská and Bírošová, *2012***) negative or positive effect of plant extracts on the frequency of spontaneous mutation of microorganisms leading to antibiotic resistance was found.

CONCLUSION

In this work, it was confirmed that some strains of enterococci isolated form poultry and pork were resistant to tested antibiotics, except vancomycin. The thyme essential oil had no antibacterial effect on enterococci at concentrations 0.033 % and 0.066 %. Thyme essential oil at concentration 0.166 % had antibacterial effect on 75% of tested enterococcal strains. No correlation between antibiotic resistance and resistance to the thyme essential oil was detected for tested enterococci.

The essential oils like thyme have the potential for use in food production as substances improving the microbiological quality and safety and also as substances extending shelf life of food products. Except experiments in vitro is very important to test the antimicrobial activity of essential oil by their application directly to the food, because the individual components as well as the properties of food can greatly influence the effect of essential oils.

The use of substances with antimicrobial activity may not always be appropriate in the fight against microbial resistance, because we do not know the mechanism of interactions (antimutagen-antibiotics, respective antimutagen-antibiotics-mutagen). Therefore, it is also necessary to continue in studies of these interactions and their influence on the development of resistance.

Acknowledgments: This work was supported by the KEGA grants from the Ministry of Education Science, Research and Sport of the Slovak Republic, grant No. 024SPU-4/2013.

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