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Volatile organic acids secreted by *Enterococcus faecium* strains

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The goal of our study was to isolate and identify volatile organic acids from the growth medium of *Enterococcus faecium (E. f.)*. The bacteria were grown in the medium made from brain–heart extract at 37 °C. Organic acids from the growth medium were isolated by water vapour distilation every 24, 48, 72, 96, 120 hours. The isolated acids were identified by the methods of thin layer chromatography and protone magnetic resonance (PMR). We found *E. f.* strain to secret formic, acetic and isobutyric acids mostly at the 96th and 120th hour of its growth. This is probably one of the mechanisms to explain the killing of pathogens.

Key words: *Enterococcus faecium*, organic acids

INTRODUCTION

Enterococci strains constitute a major part of the environment and intestinal microflora in humans and animals. The usefulness of *Enterococci* is beyond the doubt. It is more and more recognized that the resident microflora of the gastrointestinal tract plays an important role in the inhibition of gut colonization by the incoming pathogens [1]. It is known that normal flora plays the following roles: 1) competes for nutrients and receptor sites; 2) plays a role in the immunomodulation; 3) produces antimicrobial substances and organic acids [2]. Recently an idea has occurred to select bacterial strains able to incorporate into the resident flora of children, animals and demonstrating protective potentialities. The next step was to investigate their biological effects *in vitro* and *in vivo,* and finally to use them in the production of probiotics with the goal to prevent infectious diseases [3, 4]. Their modes of action have not been determined yet. Recent studies have suggested that adhesive probiotics could prevent the attachment of pathogens, such as *Salmonella spp*., *Staphylococcus aureus, Escherichia coli, Clostridium perfringens*, *Listeria monocytogenes*, and stimulate their removal from the infected intestinal tract [5–7]. However, there are very few studies, if any, on the adhesion of *Enterococci* and their inhibition of the adhesion of pathogens to the intestine. More than 60% of probiotic preparations in the market contain strains of *Enterococci* [8, 9] which in 50% consist from *Enterococcus faecium (E. f.)*. Other authors [10] reported that the same probiotic might inhibit different pathogens by different mechanisms. Probiotic *E. f.* strains 18C23, CRL35, PR88, RL produce a huge variety of antimicrobial substances including organic acids, hydrogen peroxide and bacteriocins [11–13]. One of these probiotic strains, *E. f.,* was investigated in our laboratory. Our previous results demonstrated that *E. f.* strain isolated from chicken intestine inhibited development of Salmonella *in vitro* and *in vivo* and was clinically effective in the treatment and prevention of chicken diarrhoea [14, 15]. Our present investigation is based on the isolation and identification of secreted antimicrobial component(s), particularly organic acids, and identification of the mechanism by which this strain protects the host from intestinal disorders.

MATERIALS AND METHODS

Enterococcus faecium (E. f.) consisting of the commercial probiotic preparation STF, a homogeneous, well-suspended in water powder of alive *Streptococcus faecium* cells [14]. The powder was mixed with 0.95% NaCl 1:10. The suspension was grown in a culture medium made from brain–heart extracts. The same type of agar was supplemented with 1% glucose, pH 7.0. Bacteria were grown in the medium at 38 °C and organic acids were tested every 24, 48, 72, 96 and 120 hours. The growth of bacteria was observed by changes of extracellular pH and spectrophotometrically by measuring absorption at 540 nm. Cells were precipitated by centrifugation at 6000 rpm/min for 20 min. The solution after centrifugation was filtered through a 0.22 μm nylon filter (Corning, USA). The presence of bacteria in the medium was checked by their growth in agar. The filtrate was slightly aci-

dified by conc. H_2SO_4 (indicator methyviolet) and distilled by water vapour. The distillate was neutralized with 30% NaOH (indicator phenolphthalein) and evaporated until the dry rests. The resulting salts were esterified to methyl esters. Compounds of hydroxylamine were analyzed by thin layer chromatography on the Silufol plates in a solution of butanol : acetic acid : H_2O (18:2:9). The spots were dried up and developed by 1% $FeCl_3$ solution in 96% ethanol with one drop of HCl. Another portion of salts was dissolved in deuterium oxide and analyzed by protone magnetic resonance (PMR).

RESULTS AND DISCUSSION

The results show (Fig. 1) that the growth of *Enterococcus faecium (E. f.)* cells started within 2 h of incubation. The highest density of cells was reached in 8 h of growth. We have noticed that a higher density of cells gives a lower pH: in 2 h of incubation the medium begins to acidify and in 24 h the pH reaches 4.07. The subsequent incubation gives a stable pH – 3.86–3.98. The titration results showed that the biggest amount of NaOH to neutralize the growth medium was used at 96 and 120 h (data not shown) of growth. We can conclude that organic acids were excreted mostly during that period. The next step of our study was isolation and identification of volatile organic acids. Organic acids were isolated as described in materials and methods. The results (Fig. 2) show that formic acid was excreted during the whole period of bacteria growth. The spectrum of protone magnetic resonance shows existence of more acids: formic, acetic and isobutyric acids (M. Kreneviciene, VU, spectrum not shown). The deuterium oxide and trimethylsilapropyl sodium propionate was used as a solvent and internal standard, respectively. The signal appeared at 1.32 ppm and corresponded to a $-(CH_3)_2$ group, 1.91 ppm to a –CH group, 8.45 ppm to a – CH_3 group. The molar rates of these organic acids are presented in Table. Our results showed that a higher amount of acetic acid and isobutyric acid compared to formic acid shows up at hours 48 and 120 of bacterial growth. Finally, we can conclude that during the metabolic processes of *E. f.* growth formic, acetic, and isobutyric acids were synthesized and excreted into the growth medium mostly at hours 96–120 of growth. We can compare our results with the results of other authors, even if our preparation and analysis methods are different [11]. This study is the first step to a deeper analysis of biochemical qualities of *E. f.* strain and could explain its protective activity against intestinal disorders of animals and humans.

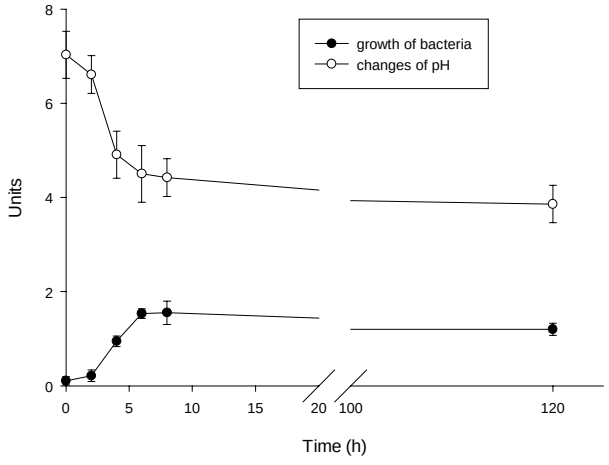

Fig. 1. Dependence of acidification of extracellular medium upon the growth of bacteria. Cells were grown and analysed as described in Materials and Methods

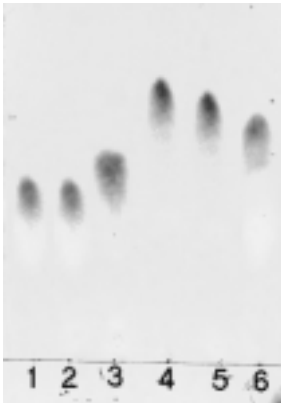

Fig. 2. Thin-layer chromatography of excreted organic acids. 1) searching sample; standards of: 2) formic acid; 3) acetic acid, 4) butyric acid, 5) valerianic acid, 6) propionic acid

Table. **The molar rates of organic acids isolated from *Enterococcus facium* and identified by protone magnetic resonance**

Strain and time of growth	Molar rates		
	Formic acid	Acetic acid	Isobutyric acid
E. f. 24 h	1	1.4	0.7
E. f. 48 h	1	1.7	0.9
E. f. 72 h	1	1.44	0.6
E. f. 96 h	1	1.37	0.59
E. f. 120 h	1	1.8	0.89

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ENTEROCOCCUS FAECIUM KAMIENŲ SEKRETUOJAMOS LAKIOSIOS ORGANINĖS RŪGŠTYS

S a n t r a u k a

Darbo tikslas – *Enterococcus faecium (E. f.)* kamieno sekretuojamų lakiųjų organinių rūgščių išskyrimas ir identifikavimas. *E. f.* kultūra auginta smegenų-širdies ekstrakto buljone 38 °C temperatūroje. Iki 8-os augimo valandos terpė tirta kas dvi valandas, vėliau – kas 24 valandas. Organinės rūgštys iš bakterijų augimo terpės išskirtos destiliuojant vandens garais ir identifikuotos plonasluoksnės chromatografijos bei protonų magnetinio rezonanso metodais (PMR). Nustatyta, kad *E. f.* kamienas 96-ą ir 120-ą augimo valandą daugiausia išskiria skruzdžių, acto ir izosviesto rūgščių, ir tuo gali būti pagrįstas vienas iš patogenų sunaikinimo mechanizmų *Enterococcus faecium* kamieno bakterijomis.