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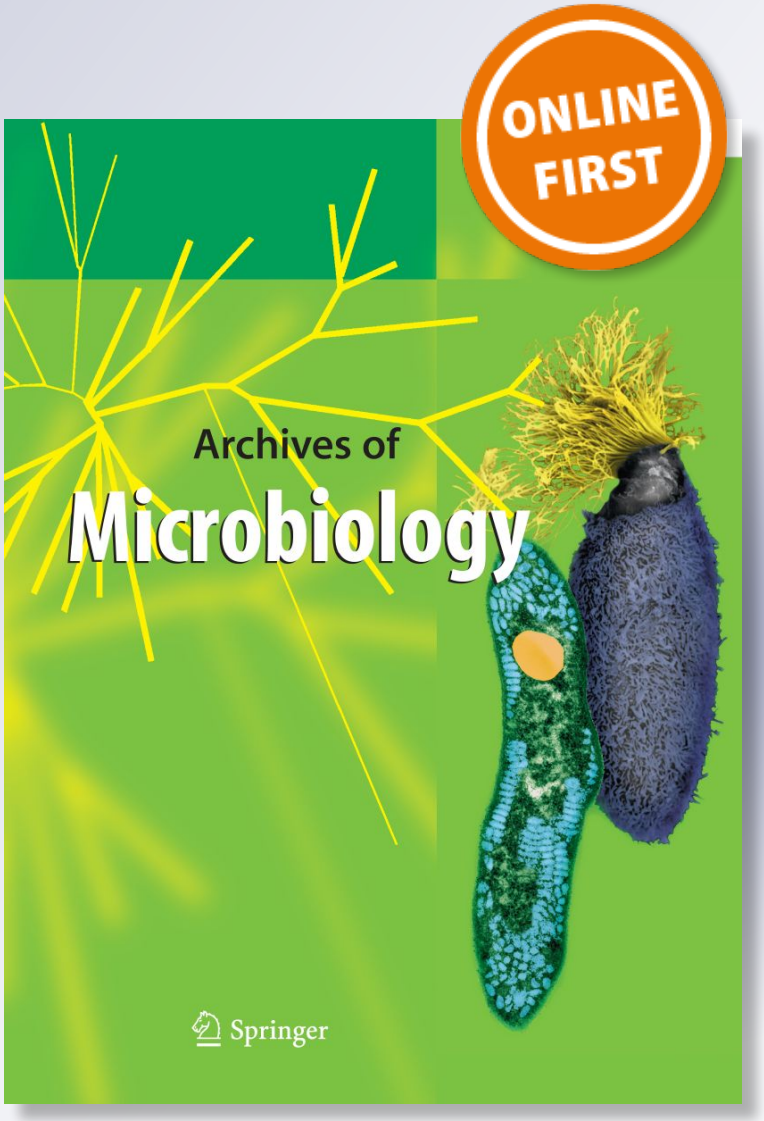

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ORIGINAL PAPER

Evaluation of the antimicrobial activity of a blend of monoglycerides against *Escherichia coli* and Enterococci with multiple drug resistance

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Abstract Bacterial antibiotic resistance is a natural phenomenon, seriously affecting the treatment of infections. The biggest danger is that current antibiotics are not able to eradicate the resistant strains. In recent years, alternative antibacterial substances are being sought, which can help in these cases. Fatty acids and monoglycerides are known among the natural substances for their antimicrobial properties and, important detail, bacteria do not develop resistance to them. In this work, we studied the antimicrobial effects of a monoglyceride blend against some multi-resistant Enterococci and *Escherichia coli* strains. Based on literature data, a blend of fatty acids and their monoglycerides was created and its antimicrobial activity was evaluated against 37 strains of *E. coli* and 17 Enterococci presenting resistance to at least two antibiotics. A different behavior was observed in the two groups of bacteria, proving that alternative substances can be considerate for the potential treatment of multidrug-resistant strains.

Keywords Multidrug resistance · Enterococci · *E. coli* · Monoglycerides · Antibacterial

Introduction

Bacterial resistance to antibiotics is a natural phenomenon, but the spreading of multidrug resistance has been worsened by their abuse and misuse in medicine and in farming (Marshall and Levy 2011; Laxminarayan et al. 2013; Woolhouse et al. 2015). The antibiotic resistance problem is even more serious when considering that the development of novel antibiotic molecules is increasingly difficult and less profitable for pharmaceutical industry (Towse and Sharma 2011; Shlaes et al. 2013). Antibiotic resistance can also represent a threat to disease control; it complicates the patient management and the treatment strategy prolonging the hospital stays (Colomb-Cotinat et al. 2016). Nowadays, this international public health problem is recognized as one of the scourges of the twenty-first century (WHO 2015). The causes of this broad diffusion of multidrug bacteria are widely accepted, for example, the overuse and inappropriate use of antibiotics for nonbacterial infections such as colds and other viral infections and inadequate antibiotic stewardship in the clinical arena and their use in animals as growth promoters that can select resistant strains (Levy 2002). Different studies have sought to estimate the morbidity and mortality of infections due to multidrug-resistant bacteria (MDRB). The European Centre for Disease Prevention and Control (ECDC 2009) published a joint report based on data from 2007, estimating at about 386,000 the annual number of infections due to MDRB in Europe and with a number of deaths associated with these infections, which was estimated at more than 25,000. In another American report by the Centers for Disease Control and Prevention (CDC 2013), it is reported an overview of the annual morbidity and mortality of antibiotic-resistant infections in the United States, estimating their number at approximately 2 million and the number of deaths associated with these infections at 23,000. These two studies underlined the importance of morbidity and mortality of antibiotic resistance on public health. Hospital environment plays a

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significant role in the occurrence of nosocomial infection since it harbors a diverse population of microorganisms (ECDC 2009). Bacterial pathogens of medical importance such as *Escherichia coli*, Enterococci, *Pseudomonas aeruginosa* and coagulase-negative Staphylococci are a common cause of healthcare-associated infection, which could be able to survive and persist for long periods in the hospital environment. Enterococci and *E. coli* are two examples of organisms present in the gut of most animals and that have emerged as nosocomial and community-acquired pathogens for their ability to develop high-level resistance to antimicrobials (Werner et al. 2012). Moreover, these bacteria are able to transfer their resistance genes by conjugation to other bacteria increasing the spread of MDRB. For these reasons, increasing interest is arising about alternative antibacterial products, especially those acting on bacterial targets, such as the bacterial membrane, that are less likely to develop mutations without hindering the survival of the bacterial cell (Gao et al. 2014). The class of compounds on which most studies are focusing are phytochemical compounds, antimicrobial peptides, and fatty acids (Hancock and Sahl 2006; Desbois 2012; Borges et al. 2015). Among the candidate replacements for antibiotics, the antibacterial activity of free fatty acids and their monoglycerides has been deeply studied by Jackman et al. (2016) since their non-specific mode of action could make them suitable for various applications not only in medicine, but also for the agri-food sector (Desbois 2012). Moreover, the development of bacterial resistance to these substances seems to be less likely in comparison with antibiotics, and it would be anyway less problematic (Desbois and Smith 2010; Schlievert and Peterson 2012). Among SCFAs and their monoglycerides, butyric acid couples antimicrobial activity with its important nutritional role for colonocytes (Namkung et al. 2011). Medium-chain fatty acids (MCFA) and their monoglycerides have also been recognized as potential antimicrobial molecules, both against Gram-positive and Gram-negative bacteria (Isaacs 2005; Batovska et al. 2009; Umerska et al. 2016). Among this group, monolaurin, the monoglyceride of lauric acid, is the most powerful compound, being strongly active against Gram-positive bacteria and enveloped viruses (Strandberg et al. 2010; Schlievert and Peterson 2012). The main problem for the application of lauric acid and monolaurin it is their poor water solubility that has been addressed using emulsifiers or developing liposomic nanocarriers or non-aqueous gels (Yang et al. 2009; Mueller and Schlievert 2015). Based on these previous studies, we created a blend (hereinafter referred to as BL) of emulsifiers, free fatty acids and monoglycerides to broaden the spectrum of antimicrobial activity and obtain a substance that could make a stable mix with water up to certain concentrations. The antibacterial activity of the blend was tested against both Gram-positive and Gram-negative bacteria, in particular different *E. coli* and Enterococci, which are among the bacteria those that easily acquire antibiotic resistances and can cause infections in humans.

Materials and methods

Bacterial strains, growth media, and chemicals

The test strains, 37 *Escherichia coli* and 17 Enterococci (11 *E. faecium* and 6 *E. faecalis*), were isolated and identified from Public Hospital Sant'Agostino, Modena (Italy). These bacteria were selected for this study because they all presented multiple antibiotic resistances. Bacteria were maintained with 20% glycerol at −18 °C and revitalized and grown for the experiments in Tryptic Soy Broth (TSB-Oxoid, Milan, Italy) for 18–24 h at 37 ± 1 °C. All compounds utilized to create the antimicrobial blend BL were purchased from Sigma-Aldrich (St. Louis, MO, USA).

GC–MS characterization of BL

The composition of the blend of SCFA, MCFA and their esters (BL) was designed on literature bases and optimized in preliminary studies (data not shown). The composition of the final substance was analyzed by gas chromatography using a gas chromatograph GC2010 PLUS (KYT, Kyoto, Japan) and a Shimadzu mass spectrometer coupled to a single quadrupole MS-QP2010 (KYT, Kyoto, Japan). GC–MS was equipped with an auto sampler HT280T (HTA srl, Brescia, Italy) to minimize operator mistakes. Samples were incubated in the auto sampler oven at 25 °C for 15 min. The injector of the GC heater was set at 270 °C. Volatile organic compounds were separated using an analytical capillary Column (5-MS capillary column, 30 m, 0.25 mm, 25 µm) and the carrier gas was ultrapure helium (99.99%) at a constant flow rate of 1.5 ml min^{-1}. The injected volume was 1 µl with a split ratio of 100:1. The temperature program for the GC was configured as follows: starting temperature 50 °C for 5 min, followed by a linear gradient 3 °C min^{-1} to 220 °C and held for 5 min, with a source temperature of 180 °C. Compounds identification was performed by NIST library (Badoni et al. 2010).

Evaluation of antibiotic resistance of clinical isolates

The antimicrobials tested were ampicillin, piperacillin, meropenem, amikacin, cefotaxime and ciprofloxacin for Gram-negative bacteria and vancomycin, ciprofloxacin, teicoplanin, tetracycline, rifampin and erythromycin for Gram-positive bacteria. Antibiotic resistance was assessed by minimum inhibitory concentrations (MICs), according

to the Clinical Laboratory Standards Institute guidelines (CLSI 2012). The resistance and sensibility to antibiotics were defined by breakpoints according to European Committee on Antimicrobial Susceptibility Testing, Version 7.1 (EUCAST 2017).

Evaluation of antimicrobial activity of BL

Antibacterial activity was evaluated on Tryptic Soy Agar (TSA- Oxoid, Milan) in which the substance was dissolved in different concentrations: (0.05, 0.1, 0.2, 0.4, 0.8%, 1, 1.1, 1.2, 1.4, 1.6, 1.8 and 2%).

The bacteria were cultured overnight at 37 °C in TSB, then spotted with micro-diluter system on the surface of culture media containing the different concentration of blend of monoglycerides and incubated for 18–24 h at 37 ± 1 °C. After incubation, the minimum inhibitory concentration of the blend was evaluated against the tested strains.

Results

GC–MS analysis of BL

The composition of BL revealed by GC–MS is reported in Table 1. The 39.2% of the mixture is composed of monoglycerides of fatty acids, namely butyric, caprylic, capric, and lauric acid (C4, C8, C10 and C12, respectively). Another 13.1% is composed by free fatty acids. The remaining 47.22% consist of glycerol and propylene glycol, used as emulsifiers.

Antibiotic resistance of selected strains

All the strains utilized in this study were resistant to at least two of the antibiotics tested. For what concerns *E. coli* isolates, five of them (13.89%) showed a double antibiotic resistance, two were resistant to three antibiotics (5.55%), thirteen strains (36.11%) possessed four resistances, 11 strains (30.56%) had five resistances and the last five (13.89%) were resistant to all six antibiotics. The same description may apply to Enterococci: 11 isolates (64.71%) showed a double resistance, two strains (11.76%) were resistant to three antibiotics, three (17.65%) had four resistances and the last one (5.88%) was resistant to five antibiotics (data not shown).

Table 1 Composition of BL by GC–MS analysis

Retention time (min)	Compound	Quantity (% w/w)
4.62	Butyric acid	8.50
6.05	Propylene glycol	13.42
8.34	Glycerol	33.80
9.69	Capric acid	1.10
9.74	Monoglycerides of butyric acid	24.40
11.14	Lauric acid	3.50
12.08	Monoglycerides of caprylic acid	2.80
13.03	Monoglycerides of capric acid	3.60
13.89	Monoglycerides of lauric acid	8.40

Antimicrobial effect of BL

The antimicrobial activity of BL resulted higher for Gram-positive bacteria than for Gram-negative bacteria. With regard to *E. coli* strains, the majority of them, 21 (58.3%) were inhibited by a concentration of 1.4% of the blend and the remaining strains were inhibited by concentrations between 1.1 and 1.6% About the Enterococci, all strains

Table 2 Minimum inhibitory concentration (MIC) of BL on the selected bacterial strains

Strain	Conc. (%)	Strain	Conc. (%)	Strain	Conc. (%)
E.c. 8	1.40	*E.c.* 104	1.40	*E. faecalis* 7	0.10
E.c. 9	1.40	*E.c.* 27	1.40	*E. faecium* 19	0.10
E.c. 12	1.40	*E.c.* 13	1.40	*E. faecium* 61	0.10
E.c. 18	1.40	*E.c.* 15	1.40	*E. faecium* 108	0.20
E.c. 25	1.20	*E.c.* 26	1.40	*E. faecium* 110	0.40
E.c. 28	1.40	*E.c.* 27	1.40	*E. faecium* 112	0.40
E.c. 29	1.40	*E.c.* 34	1.10	*E. faecium* 113	0.40
E.c. 30	1.10	*E.c.* 37	1.10	*E. faecalis* 12A	0.20
E.c. 42	1.40	*E.c.* 40	1.40	*E. faecium* A29	0.20
E.c. 53	1.10	*E.c.* 41	1.60	*E. faecalis* A30	0.20
E.c. 54	1.10	*E.c.* 43	1.40	*E. faecium* B20	0.20
E.c. 56	1.20	*E.c.* 46	1.40	*E. faecium* B5	0.40
E.c. 63	1.40	*E.c.* 60	1.40	*E. faecium* B62	0.40
E.c. 70	1.10	*E.c.* 64	1.40	*E. faecium* MR3	0.40
E.c. 95	1.10	*E.c.* 65	1.40	*E. faecalis* RO1	0,10
E.c. 96	1.10	*E.c.* 101	1.10	*E. faecalis* RO2	0,20
E.c. 97	1.40	*E.c.* 105	1.20	*E. faecalis* RO4	0,10
E.c. 98	1.20	*E.c.* 107	1.40		

E.c. Escherichia coli

were inhibited by tested concentrations between 0.1 and 0.4% (Table 2).

Discussion

It has been shown that fatty acids and their monoglycerides are effective in inhibiting bacterial growth (Namkung et al. 2011). Salsali proposed that the antimicrobial activity of organic acids could be attributed to their ability to pass across the cell membrane and dissociate in the more alkaline cell, thereby acidifying the cell cytoplasm (Salsali et al. 2008).

The BL mix, used in this study, was created with the specific aim to couple the antimicrobial properties of C4, C8, and C10 fatty acids and monoglycerides against Gram-negative bacteria (Namkung et al. 2011; Umerska et al. 2016) to the efficacy of lauric acid and monolaurin on Gram-positive bacteria, fungi, and viruses (Strandberg et al. 2010; Mueller and Schlievert 2015). In particular, the efficacy of BL was studied against different Enterococci and some *E. coli* strains. The results obtained showed that BL mix had a strong activity against Gram-positive Enterococci, while only concentrations above 1% were able to inhibit the growth of Gram-negative *E. coli* strains. Batovska et al. (2009) reported that the activity against Gram-negative species could be enhanced by increasing the percentage of MCFAs, such as caproic and caprylic acid, or coupling the activity of monolaurin with chelating cations such as EDTA or citrate. The utilization of products based on fatty acids and monoglycerides seems to be unrelated to the development of resistance against them (Schlievert and Peterson 2012) and it could even enhance the activity of traditional antibiotics against pathogens (Hess et al. 2014). The present mix or its optimized versions could be tested for their clinical application as topical treatments or, after an evaluation about the gastric protection, as food supplements.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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