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NOTE

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Abstract Terpenoids are plant metabolites which can be found in traces in the milk of animals fed with fresh forages. To these compounds, many biological properties, including antimicrobial activity, have been recognized. However, no information about the sensitivity of lactic acid bacteria (naturally occurring in milk and dairy products) to terpenoids are currently available. The Biolog methodology, which is traditionally used for the metabolic characterization of microorganisms, has also been found suitable for the evaluation of the activity exerted by plant components against bacterial consortia, allowing to establish the duration of antimicrobial activity (if present) and its resulting effect on microorganisms viability. In the present work, this approach was employed to study the effect of six oxygenated terpenoids (geraniol, linalool, alpha-terpineol, terpinen-4-ol, carvone, and menthone), which can be found in dairy products, towards 27 lactic acid bacterial strains (thermophilic or mesophilic, homo- or hetero-fermenting cocci), previously isolated from raw goat milk. Results showed that microorganisms were variously affected by the selected molecules. In some cases, terpenoids seemed to have a stimulating action; while in others, a transient antimicrobial activity was highlighted, without evident relationship with the metabolic/physiologic groups to which the tested bacterial strains belonged.

Keywords Terpenoids · Antimicrobial activity · Lactic acid bacteria · Biolog methodology

1 Introduction

Terpenoids are secondary plant metabolites that may be present, free or conjugated, in milk obtained by animals fed with fresh forages (Coulon et al. 2004). Therefore, they

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have been studied in depth and proposed as biomarkers for dairy products produced from animals fed on mountain pasture (Coulon et al. 2004; Cornu et al. 2005; Fernández García et al. 2008; Renna et al. 2012). They were also indicated as capable to indirectly affect cheeses' sensory properties due to their antimicrobial effects on autochthonous or added microorganisms, mainly lactic acid bacteria (LAB), during cheese manufacture and ripening (Mariaca et al. 1997; Buchin et al. 1999; Bugaud et al. 2001; Martin et al. 2005). However, terpenoids cannot be considered as absolute xenobiotic compounds for LAB, as these bacteria proved to be able both to synthesize and degrade such molecules (Imhof et al. 1995; Belviso et al. 2011a). These evidences suggest that a bidirectional relationship exists between terpenoids and LAB.

Raw milk, especially that collected from animals fed with fresh forages, is a typical environment in which LAB and terpenoids can occur at the same time. The way by which these two elements interact could have important outcomes on the resulting dairy products, but such investigations are not currently available. The sensitivity of LAB, selected from milk obtained by pasture-fed animals, to terpenoids, commonly found in the same type of milk, is the first step to understand the mechanisms at the basis of this relation. Due to high number of terpenoids and strains which have to be analyzed for this purpose, a method able to rapidly detect this response could be desirable.

The traditional methods for the assessment of microbial sensitivity to chemical compounds are the so-called minimum inhibiting concentration and antibiogram tests (Barry 1976; Alos and Rodriguez-Bano 2010). For both, inconvenience due to terpenes' low solubility and high volatility has been indicated. Moreover, such methods are suitable for the sensitivity assessment of a single microbial species in standard conditions, while they are less indicated for mixed populations subjected to the influence of various factors (Burt 2004). An alternative approach is represented by the Biolog methodology, a modified form of which has been developed and successfully applied to monitor the metabolic activity of total microbiota isolated from salad in presence of volatile terpenic compounds with antimicrobial activity (Bertolone et al. 2011). Moreover, by monitoring the metabolism over the whole growth curve, it is able to provide useful pieces of information about temporary and residual effect of compounds with possible antimicrobial activity on microorganisms (Bertolone et al. 2011). In the present work, the Biolog methodology has been used to study the effect of six terpenoids (geraniol, linalool, alpha-terpineol, terpinen-4-ol, carvone, and menthone) on the metabolic activity of 27 lactic acid bacterial strains (thermophilic or mesophilic, homo- or hetero-fermenting cocci) previously isolated from raw goat milk, in view to provide a contribution to the knowledge of the possible bidirectional relationship between terpenoids and LABs.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Twenty-seven lactic acid bacterial strains isolated from goat milk in previous researches have been used for the tests. These strains came from the culture collection of the Department of Agricultural, Forest and Food Sciences, University of Turin, and were all cocci (which are prevalent in goat's as in cow's milk) belonging to the

following physiologic/metabolic categories: (a) thermophilic homofermenters (*Streptococcus thermophilus*, strains I1, I2, I3, I4, and I18), (b) mesophilic homofermenters (*Lactococcus* sp. *lactis*, strains I5, I6, I7, I8, I13, I14, I15, I16, I19, I30, and I31), (c) homofermenting enterococci (*Enterococcus* sp., strains I9, I10, I11, I12, I17, and I26), and (d) heterofermenters (*Leuconostoc* sp., strains I21, I22, I23, I24, and I25). They were checked for purity and maintained on M17 agar (Oxoid, Milan, Italy). At the moment of their utilization, they were cultivated (at 30 and 40 °C for mesophilic and thermophilic strains, respectively) on BHI broth (Oxoid, Milan, Italy) and successively transferred into M17 broth (Oxoid, Milan, Italy).

2.2 Terpenoids

The following terpenoids were selected: geraniol (purity higher than 96%), (−)-linalool (purity higher than 95%), alpha-terpineol (purity higher than 99%), (+)-terpinen-4-ol (purity higher than 99%), (+)-carvone (purity higher than 98.5%), and (−)-menthone (purity higher than 99%) were purchased from Fluka (Sigma-Aldrich, Milan, Italy). Terpenoids were selected among those previously found in dairy products (Cornu et al. 2005; Belviso et al. 2011b). Moreover, on the basis of our unpublished data and as reported by other authors (Abilleira et al. 2010), the concentrations used in this work can be really found in milk.

2.3 Biolog analysis

Biolog MT2 microplates (Biolog Inc., CA, USA) with wells (120 μL capacity) containing tetrazolium violet as redox indicator were used. Terpenoid solutions were obtained by dissolving each terpenoid in absolute ethanol at the concentration of 1,000 $mg.L^{-1}$ and separately added to each well at two different final concentrations, 0.1 and 1 $mg.L^{-1}$. These values were chosen on the basis of previous studies (Abilleira et al. 2010; Bertolone et al. 2011; Belviso et al. 2011a). Cultures of each strain were taken from M17 pre-cultures at the end of the exponential phase of growth and separately inoculated at two different final concentrations, 10^3 and 10^4 $cfu.mL^{-1}$. M9 broth (Oxoid, Milan, Italy) was used to fill the well capacity.

The following blanks were also prepared: (1) microplates containing M9 broth and terpenoids; (2) microplates containing M9 broth and strain cultures.

The plates were incubated in the dark at suitable temperatures (30 and 40 °C for mesophilic and thermophilic strains, respectively) and the color changes induced in the wells by microbial activity recorded as absorbance (Ab) values by spectrophotometric readings at 590 nm on Biolog E-Max reader (Biolog Inc., CA, USA). The evolution of the microbial activity was followed for 1 week, and in particular during the first 2 days; the readings were made every 2 h to catch the lag and exponential phases, and successively, when the stationary phase was reached, every 24 h. Each assay was carried out in triplicate.

2.4 Data treatment

Ab values were converted into the index "average well color development" (AWCD) (Garland 1997). AWCD values plotted against time resulted in a curve similar to a microbial growth curve, in which the usual growth phases (lag, exponential, and

stationary) are recognizable (Fig. 1). These experimental curves were then modeled by using TableCurve 2D software (Systat Inc., city, USA) according to Gompertz's modified equation (Zwietering et al. 1990). Gompertz's modified equation is the following:

$$\mathrm{LogN} = \mathrm{LogN_0} + \mathrm{A}^*\exp(-\exp((\mu_{max}{}^*\mathrm{e}/\mathrm{A})^*(\lambda - \mathrm{t}) + 1)$$

Where N is the value of the parameter used to express the microbial growth (in this case Ab), N_0 is the same value at time zero, λ is the duration of the lag-phase, μ_{max} is the growth velocity in the exponential phase, A is the maximum value of microbial growth at the end of the exponential phase, and t is the time. Such parameters were recorded and used to quantify the effect of terpenoids on LABs.

2.5 Statistical analysis

Values of λ, μ_{max}, and A parameters of each curve were submitted to factorial analysis of variance, using strain, inoculum density, terpenoid, terpenoid concentration and their interactions as independent factors, and Duncan's test for mean comparison (Statistica for Windows ver. 7.1, StatSoft Inc., USA).

3 Results and discussion

Results of factorial analysis of variance performed on λ, μ_{max}, and A values were reported in Table 1. Parameter λ indicates the duration of the lag-phase in a growth curve (Fig. 1). For a given microorganism, at a given inoculum density, a significantly higher λ value in presence than in absence of a specific terpenoid can be considered as indicative of an antimicrobial effect exerted by that compound. On the other hand, a significantly lower value suggests that such terpenoid has a favorable effect on the metabolic activity of that particular strain. Nevertheless, such a positive or negative effect is only temporary as an exponential growth phase normally follows the lag-phase. Concerning λ values, significant differences were detected for each factor and some of their interactions (as strain/inoculum density and strain/terpenoid) (Table 1). In particular, from Duncan test (data not shown), no significant differences

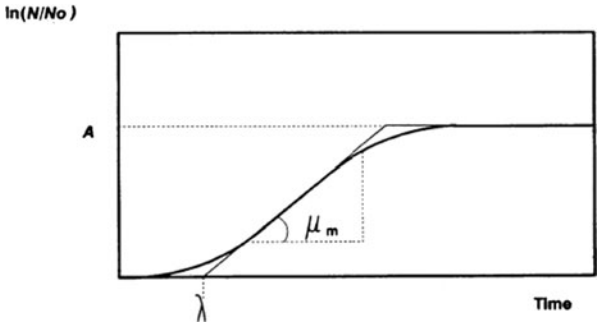

Fig. 1 A typical growth curve with the three parameters λ (lag-phase duration), μ_{max} (velocity in the exponential phase), and A (maximum value of microbial growth at the end of the exponential phase). N is the value of the parameter used to express the microbial growth, and N_0 is the same value at time zero. (original source: Zwietering et al. (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875–1881)

Table 1 Results of factorial analysis of variance performed on values of λ (lag-phase duration), μ_{max} (velocity in the exponential phase), A (maximum value of microbial growth at the end of the exponential phase) parameters, taking into account the factors strain (1), inoculum density (2), terpenoid (3), terpenoid concentration (4), and their interactions

Factors and interactions	*P*		
	λ	μ_{max}	A
(1) Strain	****	****	****
(2) Inoculum density	****	****	ns
(3) Terpenoid	****	ns	*
(4) Terpenoid concentration	*	****	*
(1) x (2)	****	****	****
(1) x (3)	****	****	****
(2) x (3)	*	*	*
(1) x (4)	*	****	*
(2) x (4)	ns	ns	ns
(3) x (4)	ns	ns	ns
(1) x (2) x (3)	ns	****	ns
(1) x (2) x (4)	ns	ns	ns
(1) x (3) x (4)	ns	ns	ns
(2) x (3) x (4)	ns	ns	ns
(1) x (2) x (3) x (4)	****	ns	****

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$; ****$P \leq 0.001$

ns not significant

resulted for most of the microorganisms tested, with the exception of strains I22, I24, I25, and I31. In fact, when inoculated with terpenoids, such strains presented a lag-phase duration significantly higher than that obtained in absence of terpenoids. The results concerning these strains are presented in Table 2 and hereby synthesized. I22 (heterofermenter) resulted sensitive to all terpenoids, at the two concentrations and inoculum densities. I24 (heterofermenter) was found sensitive to all terpenoids, with the exception of alpha-terpineol at the inoculum density of 10^3 cfu.mL^{-1}. I25 (heterofermenter) was found sensitive only to geraniol at 1 mg.L^{-1} and inoculum density of 10^3 cfu.mL^{-1}. I31 (mesophilic homofermenter) was found sensitive only to carvone at 1 mg.L^{-1} and inoculum density of 10^3 cfu.mL^{-1}. The longer duration of the lag-phase observed in presence of these terpenoids, at the concentrations and inoculum densities reported above, suggests that on the strains I22, I24, I25, and I31, a strong although temporary antimicrobial activity has been exerted.

Parameters μ_{max} and A indicate the vitality of a strain after the lag-phase. In particular, if μ_{max} and A values recorded in presence of terpenoids are higher than those registered in their absence, a stimulating effect of terpenoids on the microorganism can be suggested especially when associated to a shorter lag-phase, while if μ_{max} and A values were lower, a persisting antimicrobial action can be indicated especially in association with a longer lag-phase. On the other hand, values not significantly different from those obtained in presence of terpenoids indicate that the microorganism maintains

Table 2 Mean values of λ (lag-phase duration) for strains I22, I24, I25, and I31, of μ_{max} (velocity in the exponential phase) for strains I14, I16, I18, I19, and of A (maximum value of microbial growth at the end of the exponential phase) for strains I11 and I12, in absence of terpenoids (blank) and in presence of geraniol, linalool, alpha-terpineol, terpinen-4-ol, carvone, and menthone

			Blank	Geraniol		Linalool		Alpha-terpineol		Terpinen-4-ol		Carvone		Menthone	
	Strain			c1	c2	c1	c2	c1	c2	c1	c2	c1	c2	c1	c2
λ															
	I 22	i.d. 1	12.67	199.16	117.50	153.09	147.54	112.50	110.47	211.37	212.11	243.20	185.34	128.93	84.23
		i.d. 2	15.30	59.29	117.03	153.09	147.54	112.50	107.56	64.90	109.31	56.62	54.27	47.72	22.34
	I 24	i.d. 1	45.22	143.31	166.98	150.91	222.88	**48.42**	**44.78**	146.95	134.52	307.84	284.63	130.69	280.07
		i.d. 2	1.61	30.52	26.18	94.16	50.54	53.10	32.89	37.91	42.78	10.14	15.78	11.47	13.78
	I 25	i.d. 1	1.23	2.04	**74.88**	1.11	2.57	2.81	1.35	1.09	0.88	1.09	0.93	1.09	1.02
		i.d. 2	1.26	2.01	1.06	1.43	1.47	1.04	1.04	2.48	1.17	1.10	1.19	1.29	1.38
	I 31	i.d. 1	3.38	2.69	3.07	3.36	3.43	3.40	3.31	3.37	3.39	3.36	**90.92**	3.29	3.28
		i.d. 2	3.41	3.31	3.27	3.39	3.11	3.35	3.39	3.38	3.38	3.08	3.38	3.40	3.32
μ_{max}															
	I 14	i.d. 1	0.84	0.84	0.80	0.79	0.79	0.73	0.80	0.80	0.83	0.79	0.84	0.81	0.80
		i.d. 2	0.45	0.55	0.42	0.52	0.43	0.48	0.46	0.38	0.45	0.51	0.44	0.48	0.47
	I 16	i.d. 1	0.62	1.08	0.47	0.74	0.50	0.66	0.45	0.70	0.81	0.6	0.45	0.59	0.52
		i.d. 2	0.63	0.86	0.71	0.64	0.63	0.6	0.61	0.82	0.84	0.64	0.65	0.62	0.60
	I 18	i.d. 1	0.20	0.19	0.18	0.23	0.21	0.15	0.20	0.18	0.24	0.20	0.31	0.39	0.27
		i.d. 2	0.05	0.09	0.07	0.08	0.06	0.03	0.04	0.07	0.05	0.05	0.08	0.06	0.04
	I 19	i.d. 1	0.92	0.77	0.85	0.98	0.84	**0.08**	**0.04**	0.91	0.96	0.75	0.91	0.97	0.94
		i.d. 2	0.06	0.04	0.07	0.06	0.06	0.06	0.04	0.06	0.06	0.08	0.1	0.05	0.09
A															
	I 11	i.d. 1	19.13	14.52	20.20	**3.12**	14.98	20.15	20.10	20.12	20.23	20.51	19.97	20.15	20.17
		i.d. 2	21.77	20.61	20.29	**2.52**	21.63	21.01	21.09	19.16	21.25	19.16	21.26	21.17	21.4
	I 12	i.d. 1	7.58	8.46	7.28	8.01	7.40	8.34	7.52	8.11	8.24	8.25	**3.41**	8.17	7.70
		i.d. 2	8.70	8.51	7.14	8.97	8.14	8.45	9.10	9.60	7.98	8.36	**2.94**	9.12	9.23

i.d. 1 inoculum density at 10^3 cfu.mL^{-1}, i.d. 2 inoculum density at 10^4 cfu.mL^{-1}, c1 terpenoid concentration at 0.1 mg.L^{-1}, and c2 terpenoid concentration at 1 mg.L^{-1}

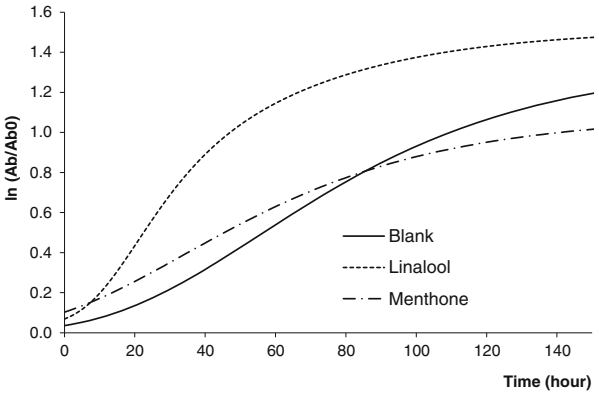

Fig. 2 A representative growth curve fitted to Gompertz modified model of I 10 strain inoculated at 10^3 cfu.mL^{-1} without terpenoid and with linalool and menthone at 1 mg.L^{-1}

its specific functionality. As an example, a representative curve (Fig. 2) is shown to see the two opposite effects on the growth phases; in the specific case, linalool has a promoting effect on the growth of the strain under study (I10), while menthone did not favor its growth especially in the exponential phase. Values of μ_{max} obtained in this work are significantly affected by all factors, except the terpenoid one, and some of their combinations (Table 1). No differences were seen for the combination in which the four factors are linked, while high significance resulted for the combination "strain/inoculum density/terpenoid." In addition, Duncan test (data not shown) highlighted that strains I14, I16, I19 (*L. lactis*), and I18 (*S. thermophilus*) cultivated in absence of terpenoids were characterized by μ_{max} values particularly high and similar to those obtained when they were cultivated in their presence with the only exception of alpha-terpineol for which a lower value was observed (strain I19 at 10^3 cfu.mL^{-1}, Table 2). Except this case, the tested terpenoids did not show antimicrobial effect on the exponential growth phase of the mentioned strains. Such finding suggests this to be a strain-linked behavior, not affected by the presence of some terpenoids.

Concerning A values significant differences within each single factor (except for inoculum density), and also for some of their combination (strain/inoculum density, strain/terpenoid, inoculum density/terpenoid, strain/terpenoid concentration, and strain/inoculum density/terpenoid/terpenoid concentration) have been picked out. In this case, the subsequent Duncan test (data not shown) showed that strains I11 and I12 (homofermenting enterococci) in presence of linalool (0.1 mg.L^{-1}) and carvone (1 mg.L^{-1}), respectively, had values significantly lower than those obtained without the addition of terpenoids (Table 2) and also lower than those obtained for the other strains under study. This seems to indicate that a residual antimicrobial effect is present.

4 Conclusions

The Biolog methodology confirmed its ability to provide useful information about the terpenoids effect on lactic acid cocci, in particular about the transience/persistence of such effect.

Some of the strains belonging to the heterofermenters resulted affected by nearly all the terpenoids tested, undergoing a delay in their metabolic activity. Also, one mesophilic homofermenting strain (I31) resulted positively affected, but only by carvone at 1 $mg.L^{-1}$ and inoculum density of 10^3 $cfu.mL^{-1}$.

Although the diffusion of such sensitivity to terpenoids within lactic acid bacteria in general cannot be established here, our results indicate that at least some of their species (mainly heterofermenting cocci) are liable to undergo a depressing effect in consequence of the presence of some terpenoids. Such effect resulted to be in general temporary, in the sense that after a certain time, practically all strains regained their "normal" growth rate, namely the one shown in the absence of terpenoids. Nevertheless, also the persistence of a residual antimicrobial effect was suggested for two *Enterococcus* sp. strains. No specific relationship was ascertained between terpenoids and physiologic/metabolic groups. This suggests the hypothesis that sensitivity to terpenoids is a "mixed" feature among lactic acid cocci that are variously (positively or negatively, temporarily or persistently) affected by various terpenoids.

Even if only temporarily, the possible antimicrobial action of terpenoids (naturally occurring in milk at the concentrations tested in this work) is likely to have no irrelevant effects on the activity of a mixed lactic acid microflora (such as the "natural" one present in some artisanal dairy products), slowing the growth of some of its components and thus favoring others, with an influence on the final characteristics of the product.

Some of the microbial strains tested (I14, I16, and I19 *L. lactis*, and I18 *S. thermophilus*) showed a metabolic activity particularly high even in presence of terpenoids. While this finding was interpreted as a strain-linked characteristic, the possibility that some lactic acid bacteria can metabolize terpenoids (with a favorable effect on their activity) cannot be excluded. Such confirmation requires further tests, which could be performed with the same Biolog methodology, but cultivating these strains with terpenoids as the only carbon source.

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