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Effects of Thymol on Ruminal Microorganisms

Jeff D. Evans, Scott A. Martin

Department of Animal and Dairy Science, 312 Animal and Dairy Science Complex, The University of Georgia, Athens, GA 30602-2771, USA

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Abstract. Thymol (5-methyl-2-isopropylphenol) is a phenolic compound that is used to inhibit oral bacteria. Because little is known regarding the effects of this compound on ruminal microorganisms, the objective of this study was to determine the effects of thymol on growth and lactate production by the ruminal bacteria *Streptococcus bovis* JB1 and *Selenomonas ruminantium* HD4. In addition, the effect of thymol on the in vitro fermentation of glucose by mixed ruminal microorganisms was investigated. Neither 45 nor 90 μg/ml of thymol had any significant effect on growth or lactate production by *S. bovis* JB1, but 180 μg/ml of thymol completely inhibited growth and lactate production. In the case of *S. ruminantium* HD4, 45 μg/ml of thymol had little effect on growth and lactate production; however, 90 μg/ml of thymol completely inhibited growth of *S. ruminantium* HD4. Thymol also decreased glucose uptake by whole cells of both bacteria. When mixed ruminal microorganisms were incubated in medium that contained glucose, 400 μg/ml of thymol increased final pH and the acetate to propionate ratio and decreased concentrations of methane, acetate, propionate, and lactate. In conclusion, thymol was a potent inhibitor of glucose fermentation by *S. bovis* JB1 and *S. ruminantium* HD4. Even though thymol treatment decreased methane and lactate concentrations and increased final pH in mixed ruminal microorganism fermentations of glucose, concentrations of acetate and propionate were also reduced.

Thymol (5-methyl-2-isopropylphenol) is a common constituent of essential oils derived from *Thymus* and *Origanum* plants and confers antimicrobial properties to these oils [5, 10, 16, 17]. In addition, this aromatic alcohol is currently used in conjunction with chlorhexidine to inhibit oral bacteria [21]. It has been postulated that thymol decreases enzyme activity and/or disrupts membrane integrity by altering protein reactions [2, 3, 15]. Previous studies have shown that thymol inhibits Gram-positive and Gram-negative bacteria, including oral selenomonads and streptococci [3, 4, 9, 15, 17].

To increase the efficiency of production, readily fermentable carbohydrates (i.e., cereal grains) are commonly incorporated into domestic ruminant diets. However, fermentation of these feedstuffs by *Streptococcus bovis* and other ruminal amylolytic bacteria leads to an accumulation of organic acids (i.e., lactate, volatile fatty acids) within the rumen and a decline in ruminal pH [8, 12, 18]. Persistence of acidic conditions within the rumen can lead to a variety of microbiological and physiological problems including death in acute cases [18]. To address these problems, ionophore antibiotics have been commonly used as feed additives in ruminant diets for over 20 years [13]. Ionophores alter the ruminal fermentation, in part, by decreasing lactate and methane production [11, 13]. To our knowledge, the influence of thymol on ruminal microorganisms has not been previously investigated. Therefore, the objective of this study was to examine the effects of thymol on growth and lactate production by *S. bovis* JB1 and *Selenomonas ruminantium* HD4. Furthermore, the effect of thymol on the in vitro fermentation of glucose by mixed ruminal microorganisms was studied.

Correspondence to: S. A. Martin; *e-mail:* scottm@arches.uga.edu

Materials and Methods

Pure culture experiments. *Streptococcus bovis* JB1 and *Selenomonas ruminantium* HD4 were obtained from J. B. Russell (USDA, Ithaca, NY). Basal medium contained, on a per liter basis, (pH 6.7): 292 mg of K_2HPO_4, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 1000 mg of Trypticase (Becton Dickinson Co., Cockeysville, MD), 1 mg of resazurin, 500 mg of yeast extract (Difco Laboratories, Detroit, MI), 28.3 mmol of acetic acid, 8.1 mmol of propionic acid, 3.4 mmol of butyric acid, and 1 mmol each of valeric, isovaleric, isobutyric, and 2-methylbutyric acids. D-

Glucose (20%, wt/vol) was prepared as a separate anaerobic solution under O_2-free CO_2, autoclaved, and added to the basal medium to achieve final concentrations shown in the figures.

Batch culture fermentations (500 ml) were conducted in gas washing bottles (Fisher Scientific, Norcross, GA) that were modified to remove or add samples through a butyl rubber stopper. The bottles were constantly purged with O_2-free CO_2, placed in a 39°C water bath, and periodically mixed. Thymol (Sigma Chemical Co., St. Louis, MO) was prepared in 97% ethanol and added to achieve final concentrations of 45, 90, or 180 μg/ml. Control incubations were also performed with an equal volume of ethanol. Samples (10 ml) were collected through the butyl rubber stopper of each gas washing bottle with a syringe and needle. All samples were immediately centrifuged (10,000 *g*, 15 min, 4°C), and the supernatants were collected and stored at −20°C.

Glucose uptake assays. *S. bovis* JB1 and *S. ruminantium* HD4 were grown in basal medium in batch culture (160-ml serum bottles), as described above, on 6 g/L D-glucose. Radiolabeled glucose uptake by whole cells was measured as previously described [6]. The effects of thymol on glucose uptake were measured by incorporating 0, 45, or 90 μg/ml thymol into the assay reaction mixture.

Mixed-culture experiments. Ruminal contents were collected from a 600-kg ruminally fistulated Hereford steer that was fed a forage diet. The ruminal contents were obtained approximately 1.5 h after feeding and were strained through four layers of cheesecloth into an Erlenmeyer flask with an O_2-free CO_2 headspace. The flask was not disturbed for 30 min while being incubated at 39°C in a water bath, permitting feed particles to rise to the top of the flask. Particle-free ruminal fluid was anaerobically transferred (20% vol/vol) to a medium (pH 6.5) containing 292 mg of K_2HPO_4, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4000 mg of Na_2CO_3, and 600 mg of cysteine hydrochloride per liter. Particle-free fluid and medium were mixed, and 40 ml was transferred anaerobically to 160-ml serum bottles that contained 10 g/L D-glucose. Thymol was prepared in ethanol and added to achieve final concentrations of 0, 50, 100, 200, or 400 μg/ml. Control incubations contained an equal volume of ethanol. The bottles were sealed (CO_2 atmosphere) with butyl rubber stoppers and aluminum seals to contain the gas pressure. The bottles were placed in a 39°C water bath and periodically mixed.

After 24 h of incubation, a gas sample (0.5 ml) was removed from each bottle and analyzed for hydrogen (H_2) and methane (CH_4) with a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a Porapak Q column (60°C, 20 ml/min of N_2 carrier gas). The bottles were then uncapped, and the pH was measured immediately with a pH meter. Bottles were then emptied into centrifuge tubes, centrifuged (10,000 *g*, 15 min, 4°C), and the cell-free supernatant fluids were stored at −20°C.

Other analyses. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) with a Beckman DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). Supernatant samples were analyzed for glucose by a coupled enzyme assay [1], and organic acids were measured by using high-pressure liquid chromatography and an organic acid column [7].

Statistical analysis. All experiments with *S. ruminantium* HD4 and *S. bovis* JB1 were performed in duplicate (n = 2) from two separate batch culture incubations. Statistical significance was evaluated with Student's *t*-test [19]. Mixed ruminal microorganism fermentations were performed on duplicate days with two experiments per day (n = 4). Data were analyzed by analysis of variance with a general linear models procedure for a completely randomized design with five concentrations (0, 50, 100, 200, or 400 μg/ml) of thymol added [20]. The group means for final pH and fermentation end products as well as control and thymol treatments were separated by using single degree of freedom contrasts.

Results and Discussion

Pure culture experiments. Initial experiments were performed to determine the effects of different concentrations of thymol on growth and lactate production by *S. bovis* JB1 (Gram positive) and *S. ruminantium* HD4 (Gram negative) (Figs. 1 and 2). When *S. bovis* JB1 was incubated in glucose medium, the cells grew rapidly and L-lactate was the primary end product that was produced (Fig. 1A). Glucose was completely fermented within 4 h, and this corresponded with a plateau in growth and L-lactate production at 4 h. Homolactic fermentation is characteristic of this bacterial species [12]. Addition of 45 μg/ml thymol prolonged the lag phase and reduced the rates of glucose utilization and L-lactate production (Fig. 1B). In the presence of 90 μg/ml thymol, glucose was not completely fermented until 5 h, and this corresponded with a plateau in growth and L-lactate production at 5 h (Fig. 1C). Both 45 and 90 μg/ml of thymol had little effect on final OD_{600} and L-lactate concentration compared with control incubations (Figs. 1B and 1C vs 1A). A thymol concentration of 180 μg/ml completely inhibited growth of *S. bovis* JB1 (Fig. 1D). Similar inhibitory effects due to thymol treatment have been reported for the oral bacterium *Streptococcus sobrinus* [14].

Selenomonas ruminantium HD4 exhibited diauxic growth that is characteristic of this strain when grown in glucose medium (Fig. 2A). During the initial 8 h of incubation, glucose was utilized and L-lactate was the primary end product. Consistent with the known physiology of lactate-utilizing selenomonads, after 8 h some L-lactate was fermented to acetate and propionate (data not shown). In the presence of 45 μg/ml of thymol, strain HD4 exhibited a slight decrease in glucose utilization and overall growth, and no L-lactate was utilized after 8 h of incubation (Fig. 2B). Growth was completely inhibited by 90 μg/ml of thymol (Fig. 2C). Thymol also inhibited growth of *Selenomonas artemidis* with a minimum inhibitory concentration of 2.0 mM (300 μg/ml) [14].

Glucose uptake. Because growth and glucose utilization were inhibited by thymol treatment, experiments were conducted to examine the effects of thymol on ^{14}C-glucose uptake by *S. bovis* JB1 and *S. ruminantium* HD4 (Table 1). Uptake by whole cells of both bacteria was inhibited ($P < 0.10$) by increasing concentrations of thymol. These results suggest that thymol disrupts mem-

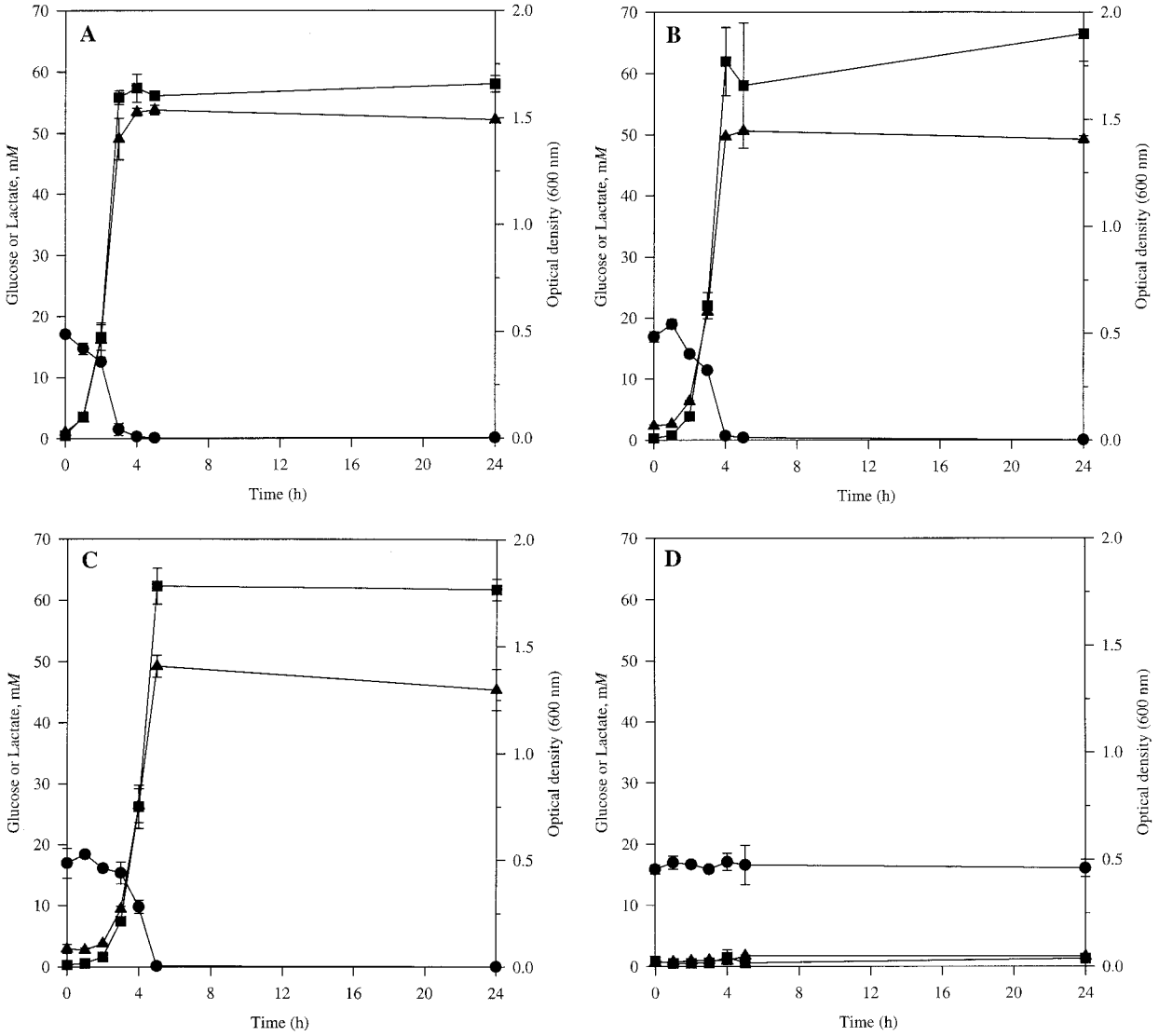

Fig. 1. Fermentation of D-glucose by (**A**) untreated cells of *Streptococcus bovis* JB1, (**B**) cells treated with 45 μg/ml of thymol, (**C**) cells treated with 90 μg/ml of thymol, and (**D**) cells treated with 180 μg/ml of thymol. Symbols represent glucose (●), lactate (■), and OD_{600} (▲). Error bars represent standard deviation (n = 2).

brane integrity in both bacteria and affects glucose transport.

Mixed ruminal microorganism fermentations. Because thymol was an effective inhibitor of lactate production by *S. bovis* JB1 and *S. ruminantium* HD4 in pure culture (Figs. 1 and 2), experiments were conducted to examine the effects of thymol on glucose fermentation by mixed ruminal microorganisms in vitro. Glucose was used to ensure that high concentrations of lactate would be produced by the mixed ruminal microorganism fermentation.

As expected, in the absence of thymol, glucose fermentation by mixed ruminal microorganisms resulted

Table 1. Effect of thymol on glucose uptake by whole cells of *Streptococcus bovis* JB1 and *Selenomonas ruminantium* HD4

	% Inhibition[a]	
Thymol (μg/ml)	*S. bovis* JB1[b]	*S. ruminantium* HD4[c]
45	0	47*
90	74*	76*

[a] Each value represents the mean of duplicate incubations (n = 2).
[b] Control specific activity values were 20.2 and 18.4 nmol/mg of protein per min for untreated and ethanol-treated cells, respectively.
[c] Control specific activity values were 25.4 and 19.6 nmol/mg of protein per min for untreated and ethanol-treated cells, respectively.
* Means differ from ethanol control value ($P < 0.10$).

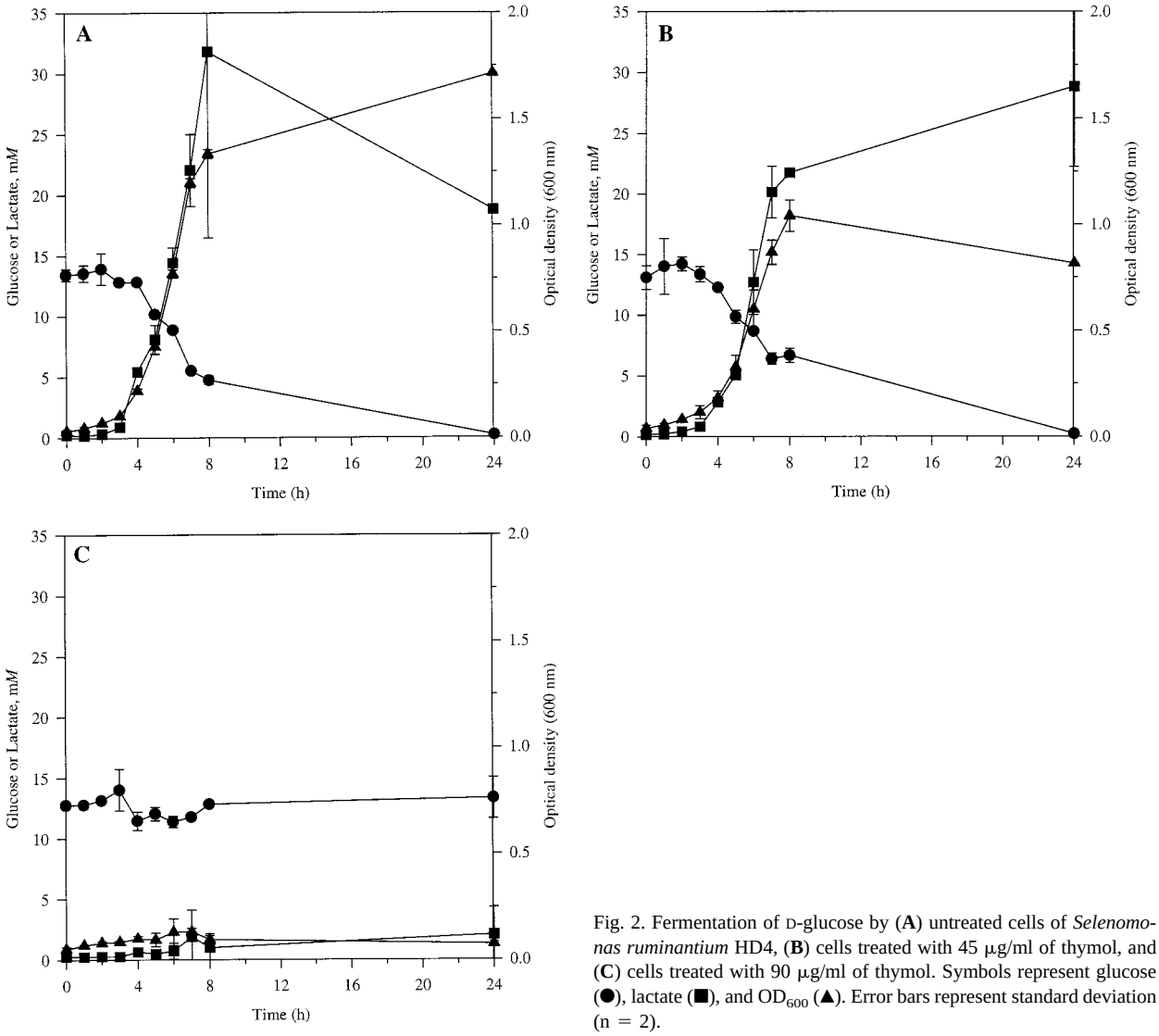

Fig. 2. Fermentation of D-glucose by (**A**) untreated cells of *Selenomonas ruminantium* HD4, (**B**) cells treated with 45 μg/ml of thymol, and (**C**) cells treated with 90 μg/ml of thymol. Symbols represent glucose (●), lactate (■), and OD_{600} (▲). Error bars represent standard deviation (n = 2).

Table 2. Effects of thymol on in vitro fermentation of D-glucose by mixed ruminal microorganisms

	Thymol (μg/ml)					
Fermentation product[a]	0	50	100	200	400	SEM[b]
pH	5.45[c]	5.50[c]	5.58[c]	5.04[c]	6.52[d]	0.11
H_2, mM	0.28	0.07	0.09	0.17	0.03	0.10
CH_4, mM	5.65[c]	6.38[c]	8.17[c]	6.11[c]	0.34[d]	0.80
Acetate, mM	19.3[c]	17.4[c]	14.9[c]	20.2[c]	10.8[d]	2.06
Propionate, mM	10.1[c]	9.2[c]	5.2[c]	7.4[c]	2.2[d]	1.32
Lactate, mM	21.5[c]	25.7[c]	25.5[c]	34.3[c]	1.4[d]	4.41
A:P ratio	1.94[c]	1.90[c]	2.86[d]	2.95[d]	5.02[d]	0.19

[a] A:P, acetate:propionate ratio.
[b] SEM, standard error of the mean.
[c,d] Means within a row lacking a common superscript letter differ ($P < 0.05$).

in high concentrations of lactate and a final pH of 5.45 (Table 2). The other primary fermentation end products included acetate, propionate, and CH_4. Addition of 50, 100, and 200 μg/ml of thymol had little effect on final pH and fermentation end products. However, 400 μg/ml of thymol increased ($P < 0.05$) final pH and decreased ($P < 0.05$) concentrations of CH_4, acetate, propionate, and lactate. The acetate:propionate ratio was increased ($P < 0.05$) by 100, 200, and 400 μg/ml of thymol.

Conclusions. Our results show that thymol was a potent inhibitor of L-lactate production by *S. bovis* JB1 and *S. ruminantium* HD4. In addition, glucose uptake by whole cells of both bacteria was inhibited by thymol. When glucose fermentation by mixed ruminal microorganisms was examined, 400 μg/ml of thymol was a strong inhibitor of CH_4 and lactate production. However, thymol treatment also inhibited acetate and propionate, and these changes in fermentation end products would not be nutritionally beneficial to the host animal.

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Literature Cited

1. Bergmeyer HU, Bernt E, Schmidt F, Stark H (1974) D-Glucose determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol III. New York: Academic Press, pp 1196–1201
2. Farag RS, Daw ZY, Hewedi FM, El-Baroty GSA (1989) Antimicrobial activity of some Egyptian spice essential oils. J Food Prot 52:665–667
3. Juven BJ, Kanner J, Schved F, Weisslowicz H (1994) Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl Bacteriol 76:626–631
4. Lis-Balchin M, Deans SG (1997) Bioactivity of selected plant essential oils against *Listeria monocytogenes*. J Appl Microbiol 82:759–762
5. Mahmoud A-LE (1994) Antifungal action and antiaflatoxigenic properties of some essential oil constituents. Lett Appl Microbiol 19:110–113
6. Martin SA (1992) Factors affecting glucose uptake by the ruminal bacterium *Bacteroides ruminicola*. Appl Microbiol Biotechnol 37: 104–108
7. Martin SA, Park CM (1996) Effect of extracellular hydrogen on organic acid utilization by the ruminal bacterium *Selenomonas ruminantium*. Curr Microbiol 32:327–331
8. Owens FN, Secrist DS, Hill WJ, Gill DR (1998) Acidosis in cattle: a review. J Anim Sci 76:275–286
9. Paster N, Juven BJ, Shaaya E, Menasherov M, Nitzan R, Weisslowicz H, Ravid U (1990) Inhibitory effects of oregano and thyme essential oils on moulds and foodborne bacteria. Lett Appl Microbiol 11:33–37
10. Pino JA, Estarron M, Fuentes V (1997) Essential oil of thyme (*Thymus vulgaris* L.) grown in Cuba. J Essent Oil Res 9:609–610
11. Russell JB (1987) A proposed mechanism of monensin action in inhibiting ruminal bacterial growth: effects on ion flux and protonmotive force. J Anim Sci 64:1519–1525
12. Russell JB, Hino T (1985) Regulation of lactate production in *Streptococcus bovis*: a spiraling effect that contributes to rumen acidosis. J Dairy Sci 68:1712–1721
13. Russell JB, Strobel HJ (1989) Effect of ionophores on ruminal fermentation. Appl Environ Microbiol 55:1–6
14. Shapiro S, Guggenheim B (1995) The action of thymol on oral bacteria. Oral Microbiol Immunol 10:241–246
15. Shapiro S, Meier A, Guggenheim B (1994) The antimicrobial activity of essential oils and essential oil components towards oral bacteria. Oral Microbiol Immunol 9:202–208
16. Shelaf LA (1984) Antimicrobial effects of spices. J Food Saf 6:29–44
17. Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T, Arsenakis M (1996) Antimicrobial and cytotoxic activities of Origanum essential oils. J Agric Food Chem 44:1202–1205
18. Slyter LL (1976) Influence of acidosis on rumen function. J Anim Sci 43:910–929
19. Snedecor GW, Cochran WG (1967) Statistical methods, 5th edn. Ames: Iowa State Univ. Press
20. Steel RGD, Torrie JH (1960) Analysis of variance I: the one-way classification. In: Principles and procedures of statistics. New York: McGraw-Hill, pp 99–128
21. Twetman S, Petersson LG (1997) Effects of different chlorhexidine varnish regimens on mutans streptococci levels in interdental plaque and saliva. Caries Res 31:189–193