Effect of *Aspergillus oryzae* Extract Alone or in Combination with Antimicrobial Compounds on Ruminal Bacteria[1]

A. A. BEHARKA[2] and T. G. NAGARAJA[3]
Department of Animal Sciences and Industry, Kansas State University, Manhattan 66506-1600

ABSTRACT

The effect of an *Aspergillus oryzae* fermentation extract on the growth rates of pure cultures of ruminal bacteria was determined. Bacteria were grown in an anaerobic ruminal fluid and carbohydrate medium. A sterile filtrate made with 10% *A. oryzae* was added to the medium at 2 or 5% (vol/vol) to provide a final *A. oryzae* concentration of 2 or 5 mg/ml, respectively. The filtrate had no effect on the growth rates of 10 of the 19 ruminal bacteria tested, however, the filtrate increased the growth rates of the bacteria that digest fiber, *Ruminococcus albus* and *Fibrobacter succinogenes*, and the bacteria that utilize lactate, *Megasphaera elsdenii*, *Selenomonas lactilytica*, and *Selenomonas ruminantium*. No differences in growth rate were detected between the two concentrations of *A. oryzae* filtrate.

We also investigated the interactions between *A. oryzae* and antimicrobial compounds on the growth rates of six species of ruminal bacteria that had shown positive responses or no response to the filtrate. The addition of *A. oryzae* filtrate to medium containing chlortetracycline or neomycin tended to diminish the negative effects of those compounds on the growth rates of some ruminal bacteria, although the bacteria had no positive growth response to the filtrate alone. In contrast, the combination of *A. oryzae* filtrate and tylosin decreased the growth rate of *Sel. ruminantium* D. These results indicated that *A. oryzae* stimulates growth of some bacteria that digest fiber and ferment lactate in the rumen and interacts positively or negatively with certain antimicrobial feed additives.

(Key words: *Aspergillus oryzae*, fermentation extract, ruminal bacteria, antimicrobial feed additives)

Abbreviation key: AO = *Aspergillus oryzae* fermentation extract.

INTRODUCTION

A wide range of microbial feed additives is commercially available to livestock producers. These additives contain microorganisms (i.e., bacteria and fungi), their products, or spent growth medium containing metabolic end products. The use of microbial additives in the diets of ruminants is increasing because these additives are an appealing alternative to antibiotics. Because microbial products are not identical in composition and because the mode of action is thought to differ among products, it is not surprising that variation in animal performance is considerable (2, 12, 30).

A fermentation extract of the mold *Aspergillus oryzae* (AO, Amaferm®, BioZyme Enterprises Inc., St. Joseph, MO) is one of several fungal products that are commercially available. Supplementation with AO increased ruminal microbial activity in a rumen simulator (9), in calves (2), and in cows (30), as was evidenced by increased VFA concentrations and bacterial numbers, particularly bacteria that digest fiber. Supplementation with AO also influences the metabolism of ruminal microorganisms. For example, AO enhanced lactate uptake by the ruminal bacteria *Selenomonas ruminantium* (19) and *Megasphaera elsdenii* (28). The effect of AO on the acetate to propionate ratio produced from mixed cultures of ruminal microorganisms has been inconsistent (9, 30), and research on the effects of AO on the growth of ruminal bacteria and their fermentation products is lacking.

Various antimicrobial feed additives, including tylosin and monensin, are fed to livestock to reduce infections and to increase performance. Microbial feed additives such as AO do not fall under FDA guidelines and, thus, can be fed in combination with any approved compound. Therefore, the determination of possible interactions between AO and antimicrobial feed additives is of interest. Our objective was to

Received July 27, 1993.
Accepted February 23, 1998.
[1]Contribution Number 92-535-J from the Kansas Agricultural Experiment Station.
[2]Present address: Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111.
[3]To whom correspondence should be addressed.

determine the effects of AO with and without antimicrobial compounds on the growth rate of pure cultures of ruminal bacteria

MATERIALS AND METHODS

Organisms

The ruminal bacterial strains examined were *Anaerovibrio lipolytica* 7553; *Bifidobacterium globosum* RU224, *Butyrivibrio fibrisolvens* 49, *Eubacterium cellulosolvens* 5495, *Fibrobacter succinogenes* S85, *Fusobacterium necrophorum* biotypes A and B, *Lachnospira multiparus* D32, *Lactobacillus ruminis* RF2, *Lactobacillus vitulinus* CL1; *Megasphaera elsdenii* B159, TS1, and LC1; *Ruminobacter amylophilus* 70 and A18, *Ruminococcus albus* 7, *Streptococcus bovis* 7H4 and JB1, *Selenomonas lactilytica* PC18, *Selenomonas ruminantium* HD4, HD1, GA31, and D, *Peptostreptococcus indolicus*, *Prevotella* (*Bacteroides*) *ruminicola* 23, and *Veillonella alcalescens* UW221 *Fibrobacter succinogenes* S85 and *R albus* 7 were obtained from American Type Culture Collection (Rockville, MD). *Fusobacterium necrophorum* biotypes A and B were isolated in our laboratory (16) Sources of the remaining strains were reported by Taylor and Nagaraja (26)

Culture Medium

All organisms were grown in a prereduced, anaerobically sterilized, ruminal fluid and carbohydrate medium, cysteine HCl (0 05%) was the reducing agent (8) The anaerobic techniques for preparing and dispensing the medium were according to the methods of Hungate (14) as modified by Holdeman et al (13)

The AO was added as a sterile filtrate to the culture medium The filtrate was prepared according to Nisbet and Martin (19) Briefly, 5 g of the AO product were mixed with 50 ml of sterile, deionized water for 1 h at room temperature (25°C) The slurry was strained through four layers of cheesecloth, centrifuged, and then vacuum filtered through a Whatman number 1 filter (Whatman Lab Sales, Hillsboro, OR) The supernatant was then filtered through a membrane filter (0 45 μm) inside an anaerobic glove box and stored inside the glove box Therefore, the final concentration of AO product in the filtrate was, at best, 10% The filtrate was added to the culture medium at 2 or 5% (vol/vol) to provide a concentration of 2 or 5 mg of AO/ml These concentrations were similar to AO concentrations used in vitro (18) and in vivo (2) Current recommended usage of Amaferm® in the diets of ruminants is between 1 and 3 g/d per head

Effects of AO on Growth Rate of Ruminal Bacteria

Screw-cap tubes (Hungate type anaerobic culture tubes, Bellco Glass Inc., Vineland, NJ) containing 10 ml of ruminal fluid medium with or without AO filtrate were inoculated with 100 μl of an overnight culture of ruminal bacteria Bacterial growth was monitored by measurement of absorbance at 600 nm in a spectrophotometer (Milton Roy, Rochester, NY) initially and at 1-h intervals until absorbance was maximal Absorbance was compared against a control sample of uninoculated medium The specific growth rate and doubling time were calculated (15) All incubations were performed in triplicate, and the experiment was replicated three times

Effect of AO on VFA Concentration

To determine the effect of AO on VFA concentration, bacterial species that exhibited increased growth rates with the addition of AO were used When the growth rate was maximal, the culture was centrifuged, and the supernatant was acidified with 25% metaphosphoric acid and analyzed for VFA by gas chromatography (2)

Interactions Between AO and Antimicrobial Compounds on Growth Rates of Ruminal Bacteria

Three bacterial species that exhibited increased growth rates with the addition of AO (*M elsdenii* B159, *Rc albus* 7, and *Sel ruminantium* D) and three species that had no response to AO (*Prev ruminicola* 23, *Rb amylophilus* 70, and *Sel ruminantium* GA31) were used to determine interactions between AO and antimicrobial feed additives on bacterial growth rates

The following compounds were included bacitracin (20 U/ml), chlortetracycline (2 5 μg/ml), monensin (10 μg/ml), neomycin (20 μg/ml), oxytetracycline (2 5 μg/ml), tylosin (2 5 μg/ml), and monensin plus tylosin Antimicrobial concentrations used were based on recommended concentrations for ruminants (3, 5, 10, 29). All antibiotics were dissolved in methanol except for chlortetracycline and oxytetracycline, which were dissolved in sterile distilled water An equivalent amount of methanol was added to the control tubes

The organisms were grown in a medium containing AO filtrate, antibiotic, AO plus antibiotic, or no addi-

tive (control) Three tubes were used for each treatment, and each tube was inoculated with 100 μl of an overnight culture grown in the same medium without antibiotic or AO Growth was monitored, and the specific growth rate and doubling time were calculated as before

Statistics

All experiments were performed in triplicate from three separate batch culture incubations Treatment means were compared by a paired sample Student's *t* test. Significance was declared at $P < 0.05$ unless otherwise indicated

RESULTS

Effect of AO on Growth of Ruminal Bacteria and VFA Concentration

Ten of the 19 species of ruminal bacteria tested (*An lipolytica* 7553, *Bif globosum* RU224, *B fibrisolvens* 49, *E. cellulosolvens* 5195, *Fs necrophorum* biotypes A and B; *L multiparus* D32, *Lb ruminis* RF2, *L vitulinus* CL1, *Rb. amylophilus* 70 and A18, *Strep bovis* 7H1 and JB1, *Sel ruminantium* GA31, *P. indolicus*, *Prev ruminicolc* 23, and *V alcalescens* UW 221) were not affected by AO treatment. However, *Fb succinogenes* S85; *M elsdenii* B159, T18, and LC1, *Rc albus* 7, *Sel lactilytica* PC18; and *Scl. ruminantium* D, HD1, and HD4 exhibited higher ($P < 0.05$) growth rates and shorter doubling times in medium containing AO than in medium without AO (Table 1). Responses did not differ ($P > 0.1$) between the 2 and 5% (0.2 and 0.5 mg/ml, respectively) concentration of AO filtrate Among *Scl ruminantium* strains, *Sel ruminantium* GA31 was unaffected by the addition of AO. None of the ruminal bacteria tested was negatively affected (decreased growth rate) by AO

The effect of AO on the VFA concentration from bacteria that showed an increased growth rate in response to AO supplementation was examined Addition of the 5% AO filtrate, but not of the 2% filtrate, increased ($P < 0.01$) the acetate concentration in the medium from *Rc albus* 7 and increased the propionate concentration in the medium from *Sel ruminantium* D compared with the control (Table 2) The increased propionate concentration accompanied by no change in acetate concentration in the medium from *Sel ruminantium* D lead to a decreased ($P < 0.01$) acetate to propionate ratio Additionally, the inclusion of AO increased VFA production ($P < 0.01$) by *Rc. albus* 7 at the 5% AO filtrate *Selenomonas ruminantium* D and HD1 exhibited a trend ($P = 0.1$) toward increased propionate and total VFA concentrations and a lower acetate to propionate ratio when AO was added to the medium The concentrations of acetate, propionate, and total VFA in the medium from *Sel lactilytica* were unaffected by the addition of AO (Table 2), although AO did increase the growth rate. The production of butyrate by *M. elsdenii* B159 increased from 6.3 to 7.2 m*M* ($P < 0.01$), and total VFA concentration from *M elsdenii* B159 (Table 2) increased, with the addition of 2%, but not 5%, AO

TABLE 1 Effect of *Aspergillus oryzae* fermentation extract (AO) on specific growth rate and doubling time of ruminal bacteria

Organism	Control		2% AO Filtrate[1]		5% AO Filtrate		SE
	Growth rate	Doubling time	Growth rate	Doubling time	Growth rate	Doubling time	
	(/h)	(min)	(/h)	(min)	(/h)	(min)	
Fibrobacter succinogenes S85	0.26[b]	155	0.35[a]	125	0.36[a]	112	0.04
Megasphaera elsdenii B159	0.32[b]	130	0.43[a]	99	0.42[a]	107	0.03
M elsdenii T81	0.30[b]	150	0.40[a]	115	0.42[a]	108	0.03
M elsdenii LC1	0.29[d]	141	0.32[c]	129	0.39[c]	117	0.01
Ruminococcus albus 7	0.58[b]	72	0.72[a]	60	0.69[a]	64	0.04
Selenomonas ruminantium D	0.59[b]	70	0.71[a]	61	0.73[a]	59	0.02
Sel ruminantium HD1	0.65[b]	66	0.75[a]	53	0.75[a]	55	0.03
Sel ruminantium HD4	0.62[b]	69	0.74[a]	54	0.72[a]	59	0.03
Sel ruminantium GA31	0.50[a]	85	0.51[a]	80	0.53[a]	74	0.05
Selenomonas lactilytica PC18	0.57[b]	73	0.72[a]	60	0.71[a]	54	0.03

[a,b]Means within a row with different superscripts differ ($P < 0.01$)

[c,d]Means within a row with different superscripts differ ($P < 0.05$)

[1]A sterile filtrate of 10% AO was added to the medium at 2 or 5% (vol/vol), providing a final AO concentration of 2 or 5 mg/ml, respectively

Additionally, the acetate to propionate ratio tended ($P = 0.12$) to increase with the addition of 5% AO. The concentrations of acetate, propionate, isobutyrate (0.39, 0.41, and 0.41 m*M* isobutyrate from medium containing 0, 2, and 5% AO, respectively), and valerate (1.5, 1.7, and 1.7 m*M* valerate from medium containing 0, 2, and 5% AO, respectively) did not change with the addition of AO to the growth medium from *M. elsdenii* B159.

Effect of AO with or Without Antimicrobial Compounds on Bacterial Growth

To determine possible interactions between AO and antimicrobial feed additives on ruminal bacteria, we grew bacteria in media containing selected antimicrobials with or without AO and compared their growth rates (Tables 3 and 4). Growth rates were measured from three bacteria (*M. elsdenii* B159, *Rc. albus* 7, and *Sel. ruminantium* D) that had shown increased growth rates and three species (*Prev. ruminicola* 23, *Rb. amylophilus* 70, and *Sel. ruminantium* GA31) that had demonstrated no change in growth rate in response to AO.

As shown previously, *M. elsdenii* B159, *Rc. albus* 7, and *Sel. ruminantium* D grew faster ($P < 0.01$) in the growth medium with AO than in the control medium (Tables 3 and 4). Growth of *M. elsdenii* B159 was unaffected by the presence of monensin in the medium (Table 3). However, *M. elsdenii* B159 grew slower ($P < 0.01$) with the addition of tylosin or monensin plus tylosin to the medium than without (control). Addition of AO to the media containing monensin, tylosin, or monensin plus tylosin did not affect the growth rate of *M. elsdenii* B159 compared with the growth rate of *M. elsdenii* B159 in medium containing the antimicrobial alone. Growth of *Rc. albus* 7 was inhibited by monensin, and the growth rate was slower ($P < 0.01$) than the growth in the control medium when tylosin or monensin plus tylosin was included. Addition of AO to media containing these antimicrobial compounds did not affect the growth of *Rc. albus* 7 (Table 3). *Prevotella ruminicola* 23 grew slower ($P < 0.01$) in media containing monensin, tylosin, or monensin plus tylosin than in the control medium. The addition of AO to the media containing monensin or monensin plus tylosin did not effect the growth of *Prev. ruminicola* 23 when compared with growth in media containing the antimicrobials alone. However, growth of *Prev. ruminicola* 23 in the medium containing tylosin plus AO was lower than that in media containing AO or tylosin alone, indicat-

TABLE 2. Effect of *Aspergillus oryzae* fermentation extract (AO) on the fermentation products of selected ruminal bacteria

Organism	AO Filtrate[1]	Acetate (A)	Propionate (P)	Total VFA	A:P
	(%)		(mM)		
Ruminococcus albus 7	0	21.2[b]		25.4[b]	
	2	22.0[b]		26.0[b]	
	5	28.9[a]		33.7[a]	
	SE	1.1		1.3	
Selenomonas ruminantium D	0	28.2	12.0[b]	40.2	2.61[a]
	2	29.6	13.8[ab]	43.4	2.11[ab]
	5	29.4	14.9[a]	44.3	2.01[b]
	SE	1.2	1.0	2.4	1.0
Sel. ruminantium HD1	0	27.8	10.1	38.3	2.71
	2	27.2	11.0	38.2	2.51
	5	27.4	12.5	39.9	2.21
	SE	1.2	1.0	2.2	1.0
Selenomonas lactilytica PC18	0	22.0	6.2	31.3	3.61
	2	24.0	6.6	33.5	3.61
	5	24.3	7.0	34.2	3.51
	SE	1.1	1.0	2.1	1.0
Megasphaera elsdenii B159	0	18.6	2.5	29.7	7.4
	2	21.8	2.8	35.3	7.9
	5	20.1	2.5	32.2	8.0
	SE	1.0	0.4	1.3	1.0

[ab]Means within a column within each organism with different superscripts differ ($P < 0.01$).

[1]A sterile filtrate of 10% AO was added to the medium at 2 or 5% (vol/vol), providing a final AO concentration of 2 or 5 mg/ml, respectively.

TABLE 3 Effect of *Aspergillus oryzae* fermentation extract (AO) with or without antimicrobial compounds on specific growth rates (per hour) and doubling times (minutes) of selected ruminal bacteria

			Treatment media					
	Control	AO[1]	Monensin (10 μg/ml)	Monensin and AO	Tylosin (2 5 μg/ml)	Tylosin and AO	Monensin and tylosin	Monensin, tylosin, and AO
Specific growth rate								
Megasphaera elsdenii B159	0 34[b]	0 43[a]	0 30[bc]	0 28[bc]	0 25[c]	0 26[c]	0 24[c]	0 24[c]
Prevotella ruminicola 23	0 42[a]	0 46[a]	0 28[b]	0 24[b]	0 24[b]	0 15[c]	0 12[c]	0 11[c]
Ruminobacter amylophilus 70	0 36[a]	0 31[a]	0 32[a]	0 30[a]	0 28[a]	0 30[a]	0 26[a]	0 27[a]
Ruminococcus albus 7	0 53[b]	0 61[a]			0 20[c]	0 21[c]	0 10[d]	0 09[d]
Selenomonas ruminantium D	0 57[b]	0 71[a]	0 56[b]	0 56[b]	0 57[b]	0 43[c]	0 52[b]	0 49[bc]
Sel ruminantium GA31	0 50[ab]	0 56[a]	0 53[ab]	0 13[cc]	0 46[bc]	0 49[ab]	0 44[c]	0 [c]
Doubling time								
M elsdenii B159	128	99	140	138	170	161	161	162
Prev ruminicola 23	122	103	165	180	181	277	401	419
Rb amylophilus 70	118	123	128	139	150	140	201	152
Rc albus 7	86	69			211	205	425	411
Sel ruminantium D	73	61	75	76	80	96	89	101
Sel ruminantium GA31	85	75	79	107	105	90	93	105

[a,b,c,d]Means within a row with different superscripts differ ($P < 0.01$)

[1]A sterile filtrate of 10% AO was added to the medium at 2 or 5% (vol/vol), providing a final AO concentration of 2 or 5 mg/ml, respectively

ing a negative interaction between the two compounds. Growth rates of *Rb amylophilus* 70 and *Sel ruminantium* D and GA31 were unaffected by monensin, tylosin, or monensin plus tylosin. The addition of AO to the medium containing tylosin did not affect the growth rate of *Sel ruminantium* GA31 compared with the growth of *Sel ruminantium* in the medium containing tylosin alone ($P < 0.01$). However, the combination of tylosin and AO caused *Sel ruminantium* D to grow slower ($P < 0.01$) than *Sel ruminantium* D in medium containing tylosin alone, indicating a negative interaction between the two compounds.

None of the bacterial species tested grew when oxytetracycline (2.5 μg/ml) was included in the medium (Table 4). *Ruminobacter amylophilus* 70 and *Prev ruminicola* 23 did not grow, and *M elsdenii* B159 and *Sel ruminantium* D and GA31 exhibited extremely slow growth rates when chlortetracycline was added to the medium compared with growth in the control medium ($P < 0.01$) (Table 4). All bacterial species tested grew slower ($P < 0.01$) in the

TABLE 4 Effect of *Aspergillus oryzae* fermentation extract (AO) with or without antimicrobial compounds on specific growth rates (per hour) and doubling times (minutes) of ruminal bacteria

			Treatment media					
	Control	AO[1]	Chlortetracycline (2 5 μg/ml)	Chlortetracycline and AO	Neomycin (20 μg/ml)	Neomycin and AO	Bacitracin (20 U/ml)	Bacitracin and AO
Specific growth rate								
Megasphaera elsdenii B159	0 34[b]	0 43[a]	0 1[e]	0 2[d]	0 19[d]	0 28[c]	0 32[bc]	0 30[bc]
Prevotella ruminicola 23	0 42[a]	0 16[a]			0 15[b]	0 17[b]	0 10[a]	0 39[a]
Ruminobacter amylophilus 70	0 36[a]	0 34[a]			0 16[c]	0 13[c]	0 22[bc]	0 30[ab]
Ruminococcus albus 7	0 53[b]	0 61[a]	0 11[d]	0 18[d]	0 13[d]	0 14[d]	0 37[c]	0 29[c]
Selenomonas ruminantium D	0 57[b]	0 71[a]	0 20[e]	0 39[cd]	0 31[d]	0 41[c]	0 47[bc]	0 48[bc]
Sel ruminantium GA31	0 50[b]	0 56[ab]	0 13[cd]	0 16[cd]	0 12[d]	0 21[c]	0 39[ab]	0 61[a]
Doubling time								
M elsdenii B159	128	99	300	246	265	164	128	148
Prev ruminicola 23	122	103			289	218	103	99
Rb amylophilus 70	118	123			347	329	196	145
Rc albus 7	86	69	375	232	338	325	113	188
Sel ruminantium D	73	61	211	107	173	146	116	93
Sel ruminantium GA31	85	75	315	261	361	197	70	65

[a,b,c,d,e]Means within a row with different superscripts differ ($P < 0.01$)

[1]A sterile filtrate of 10% AO was added to the medium at 2 or 5% (vol/vol), providing a final AO concentration of 2 or 5 mg/ml, respectively

medium with neomycin than in the control medium (Table 4) Addition of AO to the medium containing chlortetracycline or neomycin increased ($P < 0.01$) growth rates of *M elsdenii* B159 and *Sel. ruminantium* D (Table 4), however, growth rates were still slower than those in the control medium. Additionally, *Sel ruminantium* GA31 tended ($P = 0.1$) to grow faster when both neomycin and AO were added to the medium than when neomycin was added alone, even though AO alone did not affect the growth rate of *Sel ruminantium* GA31 *Ruminococcus albus* 7 and *Rb amylophilus* 70 grew slowly ($P < 0.01$) with the inclusion of bacitracin in the growth medium (Table 4). Addition of AO to the medium containing bacitracin did not affect the growth rate of either species

DISCUSSION

The use of direct-fed microbial feed additives, such as AO, in various feeding situations is increasing However, information on the mode of action of such feed additives is very limited Among direct-fed microbials, fungal products such as AO and *Saccharomyces cerevisiae* have attracted more attention than bacterial products Some strains of the fungus *A oryzae* produce substances with a wide range of antibacterial activities (20) However, none of the ruminal bacteria tested was negatively affected (decreased growth rate) by the addition of AO, suggesting that AO had no antibacterial effects on the ruminal bacteria surveyed In fact, most of the ruminal bacteria tested showed no response to AO supplementation, however, some species grew faster when AO was included in their growth medium In this study, AO incorporated in culture media was a filtered water extract of the commercial product Amaferm®. With the assumption that extraction was complete, the maximal concentration of AO added at 2 or 5% (vol/vol) was 2 or 5 mg/ml, respectively This concentration is similar to the concentration that was used by Martin and Nisbet (17) The concentration was higher than the expected concentration of AO in ruminal contents of adult cattle fed at the recommended dose of 3 g/d (18, 27)

Although a wide variety of ruminal bacteria was tested, bacteria responding to AO positively with growth represented only two functional groups, bacteria that digest fiber and bacteria that ferment lactate Among bacteria that digest fiber, only *Fb succinogenes* S85 and *Rc albus* 7 were stimulated by AO, and others (*B fibrisolvens* and *E cellulosolvens*) were unaffected The AO could have possibly provided a growth factor needed by *Fb succinogenes* S85 and *Rc albus* 7. The AO contains biotin, pantothenic acid, pyridoxine hydrochloride, vitamin B_{12}, and many amino acids (composition data from BioZyme) Second, *A oryzae* may possess some endogenous proteolytic activity (1, 3) that could lead to the increased concentration of branched-chain VFA that is sometimes reported with AO supplementation (9) Branched-chain VFA are growth factors for many ruminal fibrolytic bacteria (4, 7) However, all of the species of bacteria tested that digest fiber require biotin and pyridoxin and are stimulated by branched-chain fatty acids (4) *Fibrobacter succinogenes* is unique among the bacteria tested because it requires the straight chain acid valerate for optimal growth (4). A higher valerate concentration might contribute to a higher cell growth rate Analysis of VFA of the medium containing AO alone showed low concentrations (above the concentrations already in the medium) of branched-chain VFA (0.5 m*M* isobutyrate and 0.2 m*M* isovalerate) but no valerate Additionally, AO might have possibly enhanced nutrient uptake by the bacteria (19, 28)

Because *Fb succinogenes* and *Rc albus* are among the predominant fibrolytic bacteria in the rumens of cattle and sheep (6), the increased growth rate of these bacteria could account for the increased numbers of fibrolytic bacteria that have been reported in vitro (9) and in vivo (2) with AO supplementation Weidmeier et al (30) reported that cows fed diets supplemented with AO had higher total numbers and a greater proportion of bacteria that digest fiber than did control cows Beharka et al (2) reported increased numbers of cellulolytic, hemicellulolytic, and pectinolytic bacteria when calves were fed diets supplemented with AO compared with control calves In theory, a larger and more active population of fibrolytic ruminal bacteria can increase the rate and amount of fiber degradation However, results from digestion studies that included AO have been mixed Weidmeier et al (30) reported increased total tract digestibility of hemicellulose, but not acid detergent fiber, in response to AO Varel et al. (27) reported that AO enhanced the rate, but not the extent, of in vitro degradation of bromegrass and switchgrass fiber fractions by mixed ruminal microorganisms Additionally, Frumholz et al (9) reported that AO had no effect on in vitro DM digestion despite increased numbers of cellulolytic bacteria

In addition to increased growth rates, increased ruminal pH might also be a factor that is responsible for the increased numbers of cellulolytic bacteria in the rumen A reduction in the lactic acid concentration could lead to stabilization of the ruminal pH,

which could be accomplished by decreased lactate production or increased lactate utilization None of the ruminal bacteria that produce lactate in our study, particularly the two major lactate producers, *Strep bovis* and *Lactobacillus* species, demonstrated increased growth rates with the addition of AO In contrast, some bacterial species that are capable of utilizing lactate were stimulated by the addition of AO, including *Sel lactilytica* PC18, *M elsdenii* B159, T81, and LC1, and *Sel ruminantium* HD1, HD4, and D (Table 1) However, the growth rate of *Sel ruminantium* GA31 was unaffected by the addition of AO (Table 1) Other ruminal bacteria that ferment lactate, *An lipolytica V alcalescens*, *Fs necrophorum*, and *P indolicus*, were unaffected by AO supplementation However, *Sel ruminantium* and *M elsdenii* are the major bacteria that utilize lactate in the rumen Our results agree with results of Nisbet and Martin (19), who showed that *Sel ruminantium* HD4 grew faster with AO addition to the culture medium, and with the results of Waldrip and Martin (28), who reported growth stimulation of *M elsdenii* B159 and a selenomonad strain H18 with the addition of AO Waldrip and Martin (28) reported that growth of *M elsdenii* B159 in lactate medium containing trypticase and yeast extract was only slightly stimulated by the addition of 5% AO filtrate, however, the growth of *M elsdenii* in a similar medium lacking trypticase and yeast extract was increased twofold by the addition of AO, suggesting that AO provides growth factors, such as amino acids and B vitamins, that are required by *M elsdenii* (28) Furthermore, AO was shown to enhance lactate uptake by *Sel ruminantium* (19) and *M elsdenii* (28) The AO contains malate (19, 24), which increased the growth rate and lactate uptake by *Sel ruminantium* in vitro (19)

The increased activity of bacteria that ferment lactate may reduce lactic acid accumulation in the rumen, leading to stabilization of the ruminal pH, because lactate is a stronger acid than VFA and is often associated with low ruminal pH (25). The addition of AO to the fermentation using a ruminal simulation technique (Rusitec) reduced the postfeeding decline in pH (9) Stabilization of pH is important in ruminants fed high concentrate diets in which low pH can lead to reduced feed intake and weight gain *Megasphaera elsdenii* and *Sel ruminantium* make up a large portion of the total ruminal bacteria in cattle that consume a high concentrate diet (22) *Megasphaera elsdenii* is a producer of the branched-chain VFA and ammonia required by cellulolytic bacteria The cellulolytic bacteria, in turn, provide soluble carbohydrates to be used by bacteria that ferment lactate (4).

Concurrent with increased growth rate, the addition of AO affected VFA production by some bacteria but not by others Supplementation of AO to *Sel ruminantium* D and HD1 resulted in an increased propionate concentration and decreased the acetate to propionate ratio This result agrees with results of Nisbet and Martin (19) In contrast, *Rc albus* 7 and *M. elsdenii* 159 tended to have higher acetate to propionate ratios This result disagrees with the results of Waldrip and Martin (28), who reported AO increased valerate production only when *M elsdenii* was grown on lactate Both increased (1, 9, 30) and decreased (17) acetate to propionate ratios have been reported with AO supplementation to mixed ruminal microorganisms Several bacteria tested had increased concentrations of VFA with the addition of AO to the medium Nisbet and Martin (19) reported that AO supplementation increased production of total VFA by *Sel ruminantium* HD4. Additionally, increases in total VFA have been reported in vivo and in vitro with mixed cultures of ruminal bacteria (9, 30) The increased VFA concentrations by several ruminal bacteria with the addition of AO probably reflected increased growth rates (19)

Microbial feed additives appear to have an expanding role in the nutrition of ruminants Therefore, they are likely to be used in combination with antimicrobial feed additives, especially the ionophores Resistance or susceptibility of ruminal bacteria to antimicrobial compounds included in this study were in general agreement with other reports (5, 8, 10, 29) The exceptions were *Rb amylophilus* and *Sel ruminantium*, which have been reported to be resistant (29) but were inhibited by neomycin in our study. The addition of AO to growth media partially overcame inhibition of some bacteria by chlortetracycline and neomycin Although these two antibiotics represent different classes, both inhibit growth by binding to ribosomes and inhibiting protein synthesis (11) Why AO was able to reduce the inhibitory effects of these antibiotics in some, but not all, bacteria tested is unknown However, the binding ability of some antibiotics, such as chlortetracycline, is influenced by the ionic concentrations in the growth medium and the growth rate of the bacteria (11). The addition of AO to a medium containing monensin did not increase the growth rate of *M. elsdenii* B159 and *Sel. ruminantium* D and GA31, although they are supposedly resistant to monensin Activities of ionophores, such as monensin, are influenced by the ionic concentration of the growth medium (21) The reason

for the negative interaction between tylosin and AO is unknown

CONCLUSIONS

In conclusion, AO increased the growth rate of certain ruminal bacteria that belong to digest fiber or utilize lactate. Several ruminal bacteria showed no increase in growth rate, but none was negatively affected by the addition of AO to the culture medium Bacterial stimulation by AO may result from pH stabilization (9), enhanced nutrient uptake (19), or provision of some unknown growth factors (18, 19, 28) Undoubtedly, these factors are interrelated, and stimulation of growth rates of certain ruminal bacteria could account for increased ruminal bacterial numbers and activities in ruminants fed diets supplemented with AO. Also, AO interacted positively and negatively with certain antimicrobial compounds, suggesting that care must be taken when mixing AO with antimicrobial feed additives Therefore, further research, especially that which investigates the effects of AO and antimicrobial compounds in vivo, are warranted.

REFERENCES

1 Arambel, M J , R D Wiedmeier, and J L Walters 1987 Influence of donor animal adaptation to added yeast culture and/or *Aspergillus oryzae* fermentation extract on in vitro rumen fermentation Nutr Rep Int 35 433–436

2 Beharka, A A , T G Nagaraja, and J L Morrill 1991 Performance and ruminal function development of young calves fed diets with *Aspergillus oryzae* fermentation extract J Dairy Sci 74 4326–4336

3 Berg, J T P 1983 Enzyme production Pages 685–689 *in* Industrial Microbiology 4th ed G Reed, ed AVI Publ Co , Inc , Westport, CT

4 Bryant, M P 1973 Nutritional requirements of the predominant rumen cellulolytic bacteria Fed Proc 32 1809–1813

5 Chen, M , and M J Wolin 1979 Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria Appl Environ Microbiol 38 72–77

6 Doherty, B A 1991 Cellulose digestion in ruminants Pages 327–354 *in* Biosynthesis and Biodegradation of Cellulose C H Haigle and P J Weimer, ed Marcel Dekker, Inc , New York, NY

7 Doherty, B A , H W Scott, and P Kowaluk 1967 Volatile fatty acid requirements of cellulolytic rumen bacteria J Bacteriol 94 537–543

8 Dennis, S M , T G Nagaraja, and E E Bartley 1981 Effects of lasalocid or monensin on lactate-producing or -using rumen bacteria J Anim Sci 52 418–426

9 Frumholtz, P P , C J Newbold, and R J Wallace 1989 Influence of *Aspergillus oryzae* fermentation extract on the fermentation of a basal ration in the rumen simulation technique (Rusitec) J Agric Sci Camb) 113 169–172

10 Fulghum, R. S , B. B Baldwin, and P P Williams 1968 Antibiotic susceptibility of anaerobic ruminal bacteria Appl Microbiol 16 301–307.

11 Gale, E F , E. Cundliffe, P. E Reynolds, M H Richmond, and M J Waring 1990 Antibiotic inhibitors of ribosomal function Pages 402–547 *in* The Molecular Basis of Antibiotic Action John Wiley & Sons, London, United Kingdom

12 Gomez-Alarcon, R A , C Dudas, and J T Huber 1990 Influences of cultures of *Aspergillus oryzae* on rumen and total tract digestibility of dietary components J Dairy Sci 73 703–710

13 Holdeman, L. V , E P Cato, and W E C Moore, ed 1977 Anaerobe Laboratory Manual 4th ed Virginia Polytechnic Inst State Univ , Blacksburg.

14 Hungate, R E 1969 A roll tube method for cultivation of strict anaerobes Methods Microbiol 3 117–132

15 Koch, A L 1981 Growth measurement Pages 182–217 *in* Manual of Methods of General Bacteriology, P Gerhardt, R G E Murray, R N Costilow, E W Nester, W A Wood, N R Krieg, and G. B Phillips, ed Am Soc Microbiol , Washington, DC

16 Lechtenberg, K. F , T G Nagaraja, H W Leipold, and M M Chengappa 1988 Bacteriologic and histologic studies of the hepatic abscesses in cattle Am J Vet Res 49 59–62

17 Martin, S A , and D J Nisbet 1990 Effects of *Aspergillus oryzae* fermentation extract on fermentation of amino acids, bermudagrass and starch by mixed ruminal microorganisms in vitro J Anim Sci 68 2142–2149

18 Martin, S A , and D J Nisbet 1992 Effect of direct fed microbial on rumen microbial fermentation J Dairy Sci 78 1736–1744

19 Nisbet, D J , and S A Martin 1990 Effect of dicarboxylic acids and *Aspergillus oryzae* fermentation extract on lactate uptake by the ruminal bacterium *Selenomonas ruminantium* Appl Environ Microbiol 56 3515–3518

20 Raper, K B , and D I Fennell 1965 The Genus *Aspergillus* The Williams and Wilkins Co , Baltimore, MD

21 Russell, J B 1987 A proposed mechanism of monensin action in inhibiting ruminal bacterial growth effects on ion flux and proton motive force J Anim Sci 64 1519–1525

22 Russell, J B , and R B Hespell 1981 Microbial rumen fermentation J Dairy Sci 64 1153–1169

23 Russell, J B , W M Sharp, and R L Baldwin 1979 The effect of pH on maximum bacterial growth rate and its possible role as a determinant of bacterial competition in the rumen J Anim Sci 48 251–255

24 Sakaguchi, K , H Takahashi, and M Morino 1953 Studies on the production of α ketoglutaric acid by molds J Agric Chem Soc 23 591–595.

25 Slyter, L L 1976 Influence of acidosis on rumen function J Anim Sci 43 910–929

26 Taylor, M B , and T G Nagaraja 1987 Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives Appl Environ Microbiol 53 1620–1625

27 Varel, V H , K K Kreikemeier, H G Jung, and R D Hatfield 1993 In vitro stimulation of forage fiber degradation by ruminal microorganisms with *Aspergillus oryzae* fermentation extract Appl Environ Microbiol 59 3171–3176

28 Waldrip, H M , and S A Martin 1993 Effects of an *Aspergillus oryzae* fermentation extract and other factors on lactate utilization by the ruminal bacterium *Megasphaera elsdenii* J Anim. Sci 71 2770–2776

29 Wang, C L , B B Baldwin, R S Fulghum, and P P Williams 1969 Quantitative antibiotic sensitivities of ruminal bacteria Appl Microbiol 18 677–679

30 Wiedmeier, R D , M J Arambel, and J L Walters 1987 Effect of yeast culture and *Aspergillus oryzae* fermentation extract on ruminal characteristics and nutrient digestibility J Dairy Sci 70 2063–2068