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Ionophore resistance of ruminal bacteria and its potential impact on human health[1]

James B. Russell [a,b,*], Adam J. Houlihan [b]

[a] *Agricultural Research Service, USDA, Cornell University, Wing Hall, Ithaca, NY 14853, USA*
[b] *Department of Microbiology, Cornell University, Ithaca, NY 14853, USA*

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Abstract

In recent years, there has been a debate concerning the causes of antibiotic resistance and the steps that should be taken. Beef cattle in feedlots are routinely fed a class of antibiotics known as ionophores, and these compounds increase feed efficiency by as much as 10%. Some groups have argued that ionophore resistance poses the same public health threat as conventional antibiotics, but humans are not given ionophores to combat bacterial infection. Many ruminal bacteria are ionophore-resistant, but until recently the mechanism of this resistance was not well defined. Ionophores are highly lipophilic polyethers that accumulate in cell membranes and catalyze rapid ion movement. When sensitive bacteria counteract futile ion flux with membrane ATPases and transporters, they are eventually de-energized. Aerobic bacteria and mammalian enzymes can degrade ionophores, but these pathways are oxygen-dependent and not functional in anaerobic environments like the rumen or lower GI tract. Gram-positive ruminal bacteria are in many cases more sensitive to ionophores than Gram-negative species, but this model of resistance is not always clear-cut. Some Gram-negative ruminal bacteria are initially ionophore-sensitive, and even Gram-positive bacteria can adapt. Ionophore resistance appears to be mediated by extracellular polysaccharides (glycocalyx) that exclude ionophores from the cell membrane. Because cattle not receiving ionophores have large populations of resistant bacteria, it appears that this trait is due to a physiological selection rather than a mutation per se. Genes responsible for ionophore resistance in ruminal bacteria have not been identified, but there is little evidence that ionophore resistance can be spread from one bacterium to another. Given these observations, use of ionophores in animal feed is not likely to have a significant impact on the transfer of antibiotic resistance from animals to man.
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Keywords: Antibiotics; Ionophores; Resistance; Monensin; Ruminal bacteria

Contents

* Corresponding author. Tel.: +1 (607) 255 4508; Fax: +1 (607) 255 3904. *E-mail address:* jbr8@cornell.edu (J.B. Russell).

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1. Introduction

Throughout history man has been afflicted by bacterial infections that caused misery, a short life expectancy and periodic declines in populations (epidemics). With the discovery of penicillin by Alexander Fleming in 1928 and the development of techniques to produce antibiotics on an industrial scale, it appeared that man had finally perfected a technology that could regulate his destiny. However, in the 1950s, microbiologists began to detect bacteria that were resistant to antibiotics, and these resistances were spread from one bacterium to another on extrachromosomal elements called plasmids. By the 1980s, multiply drug-resistant bacteria were common, and the outcome of the battle between man and microbe was no longer predictable [1].

In recent years, there has been a keen debate concerning the causes of antibiotic resistance and steps that should be taken [2]. This debate has been sharply divided between two major groups: (1) physicians and veterinarians who use antibiotics therapeutically to treat acute disease and (2) livestock producers who feed antibiotics sub-therapeutically to promote (enhance) animal growth. Physicians argue that the routine use of antibiotics in animal feed creates a selection pressure for resistances that eventually spread to man. Agriculturists counter that resistance is more apt to appear when physicians and veterinarians misdiagnose infections and improperly administer antibiotics. When antibiotics are misused in this latter fashion, the dosage is greater, and the environment already has a large population of pathogens (e.g. hospitals).

Beef cattle in feedlots are routinely fed a class of antibiotics known as ionophores, and these compounds can increase feed efficiency by as much as 10% [3,4]. Ruminal bacteria resistant to one ionophore can also be resistant to other ionophores [3], but until recently the mechanism of this resistance was not well defined [5,6]. Because ionophores are technically antibiotics, some groups have called for a ban on their use and argue that ionophore resistance poses the same public health threat as conventional antibiotics [7]. We consider in this review the application of ionophores in ruminant rations, effects on ruminal fermentation, the mechanisms of ionophore activity and resistance and the potential impact of ionophore resistance on human health.

2. Use of antibiotics in animal feed

After World War II, antibiotic production developed for military use continued, and the industrial residues were eventually fed to farm animals [8]. Feeding trials indicated that poultry fed *Streptomyces aureofaciens* biomass grew more efficiently, and this benefit was initially attributed to vitamins (hypothetically vitamin B_{12}). When purified chlortetracycline gave a similar response, the vitamin hypothesis was abandoned [8]. The amount of antibiotic that is added to animal feed to promote growth is typically 5–10-fold lower than the amount that is used to combat clinical disease [9].

The mechanism of antibiotic growth promotion has never been clearly defined [10], but the activity is not highly specific, and many antibiotics have enhanced either animal growth or feed efficiency [9]. Because the magnitude of animal growth promotion is inversely related to the degree of sanitation in the animal's environment, it appears that antibiotics are able to suppress sub-clinical populations of potentially harmful bacteria [10]. Improvement in growth efficiency by antibiotic use is less than 10%, but the economic benefit of antibiotic use can be as great as the profit margin of production.

Antibiotics were first used to promote the growth of chickens, but they were eventually fed to swine, beef cattle and even dairy cattle [9]. The use of antibiotics in dairy cattle feed was suspended when it became apparent that milk contamination was a serious problem. Contaminated milk cannot be used for cheese making, and some humans had allergic reactions to antibiotics in milk.

The United States pioneered the use of antibiotics in animal feed, but until recently chickens, swine and beef cattle throughout the world were fed antibiotics [7]. In recent years, the scale and magnitude of animal production has been greatly increased, and it is conceivable that antibiotics could have a prophylactic effect on the spread of disease from one animal to another [10]. Because university and government trials are typically conducted with small groups of animals and the experimental period is usually short, the impact of a widespread ban on antibiotic usage has not been thoroughly assessed.

In the 1970s, the U.S. Food and Drug Administration (FDA) approved a new class of antibiotics (ionophores) for cattle. The ionophore, monensin, was originally developed as a coccidiostat for chickens [3], but Richardson et

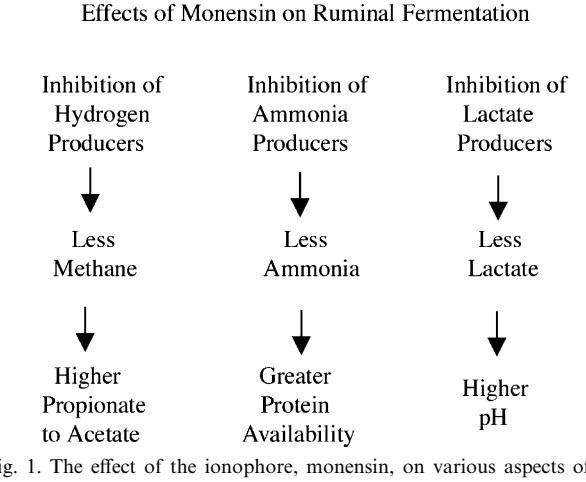

Fig. 1. The effect of the ionophore, monensin, on various aspects of ruminal fermentation. Taken with permission from J.B. Russell (2002) Rumen Microbiology and Its Role in Ruminant Nutrition, p. 88. J.B. Russell Publ. Co., Ithaca, NY.

al. [11] and Dinius et al. [12] showed that monensin increased the feed efficiency of cattle and sheep via a mechanism that altered ruminal fermentation (Fig. 1). Monensin-treated ruminants produced less methane, had a lower ratio of ruminal acetate to propionate and had a greater feed efficiency [13]. Later work showed that monensin decreased ammonia, a wasteful end-product of protein degradation [3]. Monensin also seems to modulate food intake and increase the ruminal pH of cattle fed large amounts of grain [14,15]. Cattle fed lush legumes or large amounts of starch sometimes bloat and some ionophores have been effective in counteracting this disorder [16].

Streptomyces produce many different ionophores, and the FDA has approved two other ionophores for beef cattle (lasalocid and laidlomycin). Nonetheless, monensin, produced by *Streptomyces cinnamonensis*, is still the most widely used ionophore [17]. In feedlot cattle, monensin-dependent improvements in feed efficiency are typically 6%, and the efficacy can be as much as 10 to 1 [4]. Monensin is also fed to grazing cattle, and the increase in average daily gain is 15% [4]. Based on annual ionophore sales of more than $100 million, the benefit of ionophore to the cattle industry could be as much as $1 billion per year (Leo Richardson, Elanco Corp., personal communication).

Ionophores are also used extensively in Australia, New Zealand and Latin America, but ionophore use for cattle in Europe was never great [10]. The tendency of European farmers not to use ionophores stems in large part from small herd sizes and the dual purpose of their cattle (milk and meat production). New Zealand dairy farmers use ionophores to increase the milk production of dairy cattle and prevent bloat, but this use is monitored by veterinarians that issue a prescription. Elanco has filed an application for lactating dairy cattle in the United States, but the FDA has not yet approved this application (Leo Richardson, Elanco Corp., personal communication).

3. Mechanism of ionophore action

Antibiotics inhibit various aspects of bacterial growth and replication, and these targets include peptidoglycan synthesis (penicillins, cephalosporins, imipenem, aztreonam, vancomycin, cycloserine and bacitracin), ribosome activity (chloramphenicol, erythromycin, clindamycin, tetracyclines and aminoglycosides), DNA replication (quinolones), mRNA transcription (rifampin), nucleotide synthesis (sulfonamides and trimethoprim) and membrane stability (polymixin) [18]. Ionophores also affect cell membranes, but they have a distinctly different mechanism of action from polymixin [19].

Ionophores are highly lipophilic polyethers that accumulate in cell membranes and catalyze rapid ion movement [19]. Some ionophores (e.g. valinomycin) only move a single ion, but ionophores fed to cattle act as antiporters [3]. Antiporters bind protons or metal ions (e.g. sodium and potassium), and only uncharged molecules containing either a proton or metal ion can move freely through the cell membrane (Fig. 2). Metal ion binding is facilitated by the loss of solvation water and the ability of the linear molecule to form a 'donut' and shield this charge [20]. Because the carboxyl group of monensin remains near the surface, and its ionization is a pH-dependent function, ionophores such as monensin and lasalocid are often more effective when the pH is low [21]. If the pH is higher than the pK_a of the antiporter, the ionophore is largely ionized, and its ability to dissipate ion gradients is decreased. However, if the pH is lower than pK_a, a large fraction of the ionophore penetrates the cell membrane, the proton is released to pick up a metal ion, and the ionophore travels across the cell membrane to catalyze a futile ion cycle (Fig. 2). Monensin has a pK_a of 7.95 and lasalocid has a pK_a of 5.8 [22].

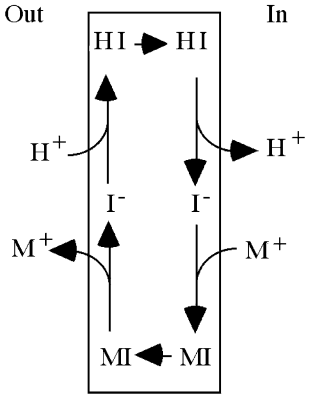

Fig. 2. The ability of an ionophore (I) to translocate protons (H^+) and metal ions (M^+) across the cell membranes of sensitive bacteria. Taken with permission from J.B. Russell (2002) Rumen Microbiology and Its Role in Ruminant Nutrition, p. 89. J.B. Russell Publ. Co., Ithaca, NY.

Ionophores have different 'selectivities', but the direction of metal and proton movement is ultimately dictated by the magnitude of ion gradients across the cell membrane [3] in accordance with the Nernst equation: $Z\log_{10}[X^+]_{in}/[X^+]_{out}$, where Z has the value of 62 mV at 39°C, and X^+ denotes an ion that is translocated across the cell membrane to establish a concentration gradient. Most living organisms maintain a higher concentration of potassium inside than outside their cells, and they expel sodium and protons [23]. The rumen is an 'inland sea' that is rich in sodium, and ruminal sodium concentrations are typically 2–10-fold greater than potassium concentrations [24]. When glycolyzing *Streptococcus bovis* cells were treated with monensin [3,25], there was a rapid efflux of potassium and an influx of sodium and protons (Table 1). Because the cells attempted to counteract futile ion flux by activating membrane ATPases and transporters, they were eventually de-energized (Fig. 3).

Ionophores can also translocate ions across the cell membranes of mammals, and this characteristic limits

their therapeutic use [19,22]. Some animals can tolerate ionophore doses needed to inhibit sensitive bacteria, but this tolerance is highly species-specific. Liver enzymes that degrade and inactivate parent compounds and the ability of the enterohepatic circulation to recycle absorbed ionophore back to the gut via the bile mediate the ionophore tolerance of cattle [26]. Work with horses indicates that relatively small doses of lasalocid or monensin cause depression, ataxia, paresis, paralysis, anorexia, and death [27,28]. The effect of ionophores on humans has not been experimentally determined. However, Pressman [19] noted that people exposed to monensin during its manufacture had symptoms including headache, nausea, nose bleed and skin rash, and ranchers that fed monensin to cattle had headache and dizziness.

4. Effect of ionophores on ruminal bacteria

In vitro and in vivo experiments indicate that monensin decreases methane production, but methanogenic bacteria are not particularly sensitive to ionophores [29]. When mixed ruminal bacteria are incubated with H_2 and CO_2, little decrease in methane is observed [29], but monensin can inhibit H_2-producing ruminal bacteria that supply methanogens with H_2 [30]. Because bacteria that produce H_2 are more apt to produce acetate and butyrate than those that produce succinate and propionate, there is a change in ruminal fermentation acids (Fig. 1). The ability of ionophores to decrease methane and shift the fermentation from acetate to propionate only explains one-third of the potential increase in feed efficiency [3]. Ionophores decrease methane production by 30%, but methane production never accounts for more than 12% of the feed energy [31].

Feeding trials [32] and in vitro incubations [29,33] indicated that monensin could decrease wasteful amino acid deamination, but previously isolated ammonia-producing ruminal bacteria were, in most cases, highly monensin-resistant [34]. In the 1980s ruminal bacteria with 20-fold higher rates of ammonia production were isolated, and these obligate amino acid-fermenting bacteria were monensin-sensitive [35,36]. Most obligate amino acid-fermenting bacteria are closely related to clostridia, but Attwood et al. [37] isolated one strain that was more closely related to *Fusobacterium necrophorum*. When cattle were fed timothy hay, monensin caused a 40% decrease in ruminal ammonia, and this decrease could be explained by an inhibition of deamination [38]. Cattle not fed monensin had a specific deamination rate of 27 nmol ammonia mg protein^{-1} min^{-1}. However, those given 350 mg monensin each day had a rate of only 17 nmol ammonia mg protein^{-1} min^{-1}, and they had 10-fold fewer, obligate amino acid-fermenting bacteria. The amino acid sparing effect of ionophores is, however, diet-dependent. When cattle were fed alfalfa and a similar dose of monensin, the specific deamination rate decreased 30%, but the steady-state concentration of ruminal ammonia did not decline [39].

Beef cattle are often given large amounts of cereal grain to fatten them, but these rations can cause ruminal ulcers, metabolic acidosis, founder and even death of the animal [40]. Acute ruminal acidosis is caused by *S. bovis*, a lactic acid-producing bacterium that grows faster than other ruminal bacteria [41]. In vitro experiments demonstrated that *S. bovis* could be inhibited by monensin (Fig. 1), but in vivo doses could not counteract the explosive proliferation of *S. bovis* in animals given large amounts of glucose or starch [14].

Discrepancies between in vitro and in vivo results are related to differences in bacterial mass and the ability of ionophores to bind to other materials. Because the rumen volume of cattle is approximately 70 l and the recommended daily dosage of monensin is 350 mg, and the molecular mass of sodium monensin is 693, researchers often

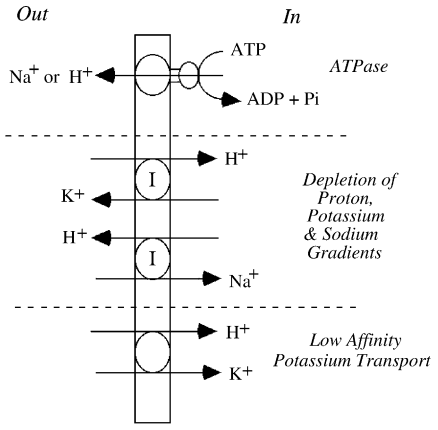

Fig. 3. The ability of an ionophore (I) to translocate protons (H^+), potassium ions (K^+), sodium ions (Na^+) across the cell membranes of sensitive bacteria and promote ATPase activity and potassium transport. Taken with permission from J.B. Russell (2002) Rumen Microbiology and Its Role in Ruminant Nutrition, p. 89. J.B. Russell Publ. Co., Ithaca, NY.

Table 1
The effect of monensin on the membrane potential and intracellular cations of monensin-sensitive *S. bovis* cells[a]

Measurement	Control	Monensin
pH_e[b]	6.65	6.65
pH_I[b]	7.08	6.20
$Z\Delta pH$ (mV)[b]	−26	+28
$\Delta\Psi$ (mV)[b]	77	71
Δp (mV)[b]	103	43
K_e (mM)[b]	9	9
K_i (mM)[b]	613	134
Na_e (mM)[c]	89	93
Na_i (mM)[b,c]	16	322
ATP (mM)[d]	3.2	1.8

[a]Washed cell suspensions were energized with glucose and treated with 5 μM monensin for 15 min (39°C).
[b]Taken from Russell [25].
[c]Taken from Strobel and Russell [97].
[d]Russell, unpublished results.

used an in vitro concentration of 5–10 μM [11,14,21,29,42]. However, ionophores accumulate in cell membranes and bacterial mass in vitro is typically 10-fold lower than in vivo. Studies using ^{14}C monensin and ^{14}C lasalocid showed that ionophores bind to feed particles, protozoa and ionophore-resistant bacteria as well as ionophore-sensitive ones [43].

Ionophores do not inhibit all ruminal bacteria, but there is often a decrease in total feed intake [11,12]. When feed intake decreases, the microflora has a longer time to digest feed materials, and total tract digestibility has in some cases increased [12]. However, ionophores do not affect all components of the diet in the same fashion. Ionophores do not decrease starch digestion [44], but in vitro and some in vivo experiments indicate that cellulose digestion may be inhibited [45,46].

The rumen has a complex population of protozoa, and protozoa can account for as much as 50% of the microbial protein in the rumen [47]. The protozoa are not essential and can be eliminated by rearing ruminants in isolation or by treating them with harsh chemicals. In vitro studies indicated that ruminal protozoa were sensitive to monensin [48], but in vivo studies indicated that protozoal counts were not depressed [12]. The rumen also has a small population of fungi that are sensitive to monensin in vitro [49], but these organisms are only important if the diet is extremely fibrous [50].

5. Mechanisms of ionophore resistance

Bacteria that produce ionophores are naturally resistant, but the mechanism of this resistance has not been well defined. Linton et al. [51] located the tetronasin resistance determinants of *Streptomyces longisporoflavus* and

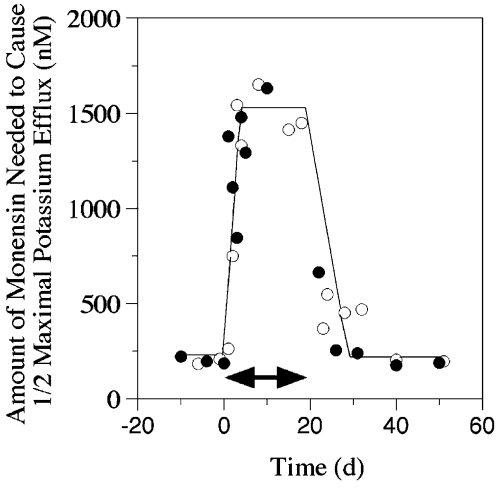

Fig. 4. The effect of monensin (applied over the time indicated by the arrow) on the amount of monensin that is needed to cause half-maximal potassium depletion from mixed ruminal bacteria. The cows were fed a diet consisting of timothy hay. Taken with permission from Lana and Russell [60].

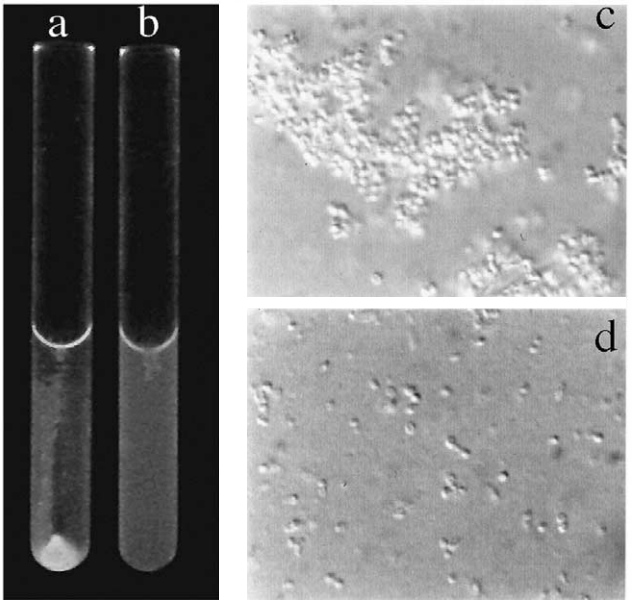

Fig. 5. The ability of lysozyme to agglutinate wild-type (a,c) and monensin-selected (b,d) *P. bryantii* B_14 cells. Taken with permission from Callaway and Russell [6].

cloned them into *Streptomyces lividans*. Sequence homology suggested that one of the genes was an ABC-transporter, and they concluded that resistance might be mediated by an ATP-efflux system. Ionophores can also be degraded by aerobic bacteria and mammalian enzymes, but these pathways are oxygen-dependent and not functional in anaerobic environments like the rumen or lower GI tract [26].

In vitro and in vivo experiments indicate that only some ruminal bacteria are inhibited by ionophores. Bergen and Bates [52] noted that monensin-resistant ruminal bacteria often have membrane-bound fumarate reductase activity, and they hypothesized that this proton-translocating enzyme might counteract ionophore-dependent ion flux. Morehead and Dawson [53] noted that monensin-resistant *Prevotella ruminicola* strains produced more propionate and appeared to have more fumarate reductase activity than those that were monensin-sensitive, but at least one fumarate reductase-containing ruminal bacterium (*Ruminococcus flavefaciens*) is highly sensitive to monensin [30].

Ionophore sensitivity and resistance of ruminal bacteria is more closely correlated with differences in the cell envelope [45,54]. Gram-positive bacteria are in many cases more sensitive to monensin than Gram-negative species, and they are more apt to produce acetate, butyrate and hydrogen (Fig. 1). The cell wall model of monensin resistance is, however, not always clear-cut. 16S rDNA sequencing indicates that *Selenomonas ruminantium* and *Megasphaera elsdenii* have outer membranes and are highly ionophore-resistant even though they are closely related to Gram-positive ruminal bacteria [55].

Some Gram-negative ruminal bacteria are initially ionophore-sensitive and need a period of adaptation to be-

come resistant [30]. Potassium efflux measurements indicated that monensin-adapted *Prevotella bryantii* $B_1$4 cultures were 16-fold more resistant than those that were not selected with monensin, but the original culture was a mixture of monensin-sensitive and -resistant cells [5]. When monensin was withdrawn from the culture, sensitive cells displaced the resistant ones. A comparison of 15 *Prevotella* strains indicates that monensin sensitivity could vary by as much as 100-fold, but only some strains could adapt significantly [56].

S. bovis is a Gram-positive ruminal bacterium that has often been used as a model of monensin sensitivity [3,57], but Dawson and Boling [58] described a *S. bovis* strain (S1) that grew in the presence of 58 μM monensin. The S1 strain did not retain this phenotype [21], but *S. bovis* JB1 cultures that were selected with monensin were eight-fold more resistant than those that were not treated with sub-lethal doses [55]. *Clostridium aminophilum* is a Gram-positive, amino acid-fermenting ruminal bacterium that can be inhibited by monensin in vitro [36], but physiological doses could not eliminate it from the rumen [59]. Unadapted *C. aminophilum* was inhibited by 1 μM monensin, but these cultures eventually grew and could not be inhibited a second time [6].

Recent research indicates that extracellular polysaccharide plays a key role in the ionophore resistance of ruminal bacteria (Fig. 5). When *P. bryantii* $B_1$4 [5] and *C. aminophilum* F [6] cultures were selected with monensin, the monensin-resistant cells were: (1) more easily dispersed, (2) had an increased amount of anthrone-reactive material, and (3) were no longer agglutinated by lysozyme, a positively charged protein. Because the resistant cells did not persist after the ionophore was withdrawn, there was little indication that resistance was mediated by a traditional mechanism (e.g. a degradative enzyme or a pump that expelled antibiotics). Little is known about the genetics of extracellular polysaccharide production in ruminal bacteria, but studies with non-ruminal bacteria indicate that it is encoded by a large number of inducible genes [60].

Antibiotic sensitivity is generally assessed with free-living (planktonic) cells, but naturally occurring bacteria often exist in biofilms. Cells in biofilms grow slowly, have more extracellular polysaccharide (glycocalyx) and are more resistant to therapeutic antibiotics than planktonic cells [61], even if they do not have plasmids, transposons or mutated cellular targets [62]. For example, a biofilm of *Klebsiella pneumoniae* was more resistant to ampillicin than free-living cells, but it was β-lactamase-negative and lacked other traditional mechanisms of antibiotic resistance [63]. The use of antibiotics to treat infectious biofilms is further complicated by bacteria called 'persisters' [64]. Persisters have altered growth characteristics. Because they grow slowly and do not react with antibiotics in the normal suicidal fashion, they can re-populate the biofilm once the antibiotic is withdrawn.

Some workers have explained the increased antibiotic resistance of biofilms by the ability of bacterial glycocalyx to act as a permeability barrier [64], but the binding of ions to this matrix is a reversible process that is highly dependent on the nature of the molecule (e.g. size, charge distribution and hydrophobicity). Spoering and Lewis [65] concluded that bacterial growth rate might be a more important characteristic than glycocalyx per se. Because bacteria in biofilms grow very slowly, they have slower rates of protein synthesis and cell wall expansion than faster-growing ones. It has long been recognized that slow-growing cells are more resistant to most antibiotics, and this difference provides a basis for (e.g. penicillin) counter-selection [66].

Most ruminal bacteria are attached to feed particles [67], but this community is distinctly different from medical biofilms. Attached ruminal bacteria secrete enzymes that degrade the macromolecules (e.g. cellulose, hemicellulose, pectin, starch, proteins, etc.), and this degradation produces substrates that are subsequently utilized for growth. Because feed materials are only retained in the rumen for short periods of time, the rate of rumen bacterial growth is relatively rapid, and ruminal 'biofilms' have a short half-life [68,69].

The impact of bacterial growth rate on ruminal ionophore resistance has not been examined in a systematic fashion, but continuous culture studies with mixed and pure cultures indicate that slow-growing cells are not necessarily more resistant than batch cultures. Continuous culture studies with *P. bryantii*, a bacterium that can be adapted and selected to grow with very high concentrations of monensin, indicated that slow-growing cells were two-fold more sensitive to monensin than those that were grown in batch culture [5]. When Wallace et al. [70] treated mixed ruminal microflora in an artificial continuous culture device, monensin-dependent fermentation changes were observed almost immediately after the ionophore was added, and there was no indication of long-term adaptation. Given these observations, it appears that traditional models of 'biofilm' resistance are not applicable to the rumen.

6. Dissemination of ionophore resistance

Early work suggested that some bacteria were not susceptible to therapeutic antibiotics, but the significance of antibiotic resistance was not initially a major concern [1]. However, during an outbreak of bacterial dysentery in 1955, Japanese physicians isolated a strain of *Shigella dysenteriae* that was resistant to four antibiotics (sulfanilamide, streptomycin, chloramphenicol and tetracycline), and by 1964 half of all Japanese *Shigella* infections were caused by multi-drug-resistant variants [1]. These results indicated that antibiotic resistance evolved quickly and could be easily disseminated.

Because antibiotic resistances are often produced constitutively [71], it had been assumed that antibiotic-resistant bacteria would disappear from the environment soon after treatment was suspended [72]. However, later work indicated that even constitutively expressed genes could persist for long periods in the absence of antibiotics, and these observations contradicted the assumption that antibiotic-resistant bacteria would be at a disadvantage if antibiotic was not present [73]. The persistence of antibiotic resistance genes has complicated the use of antibiotics as human therapy.

The transfer of antibiotic-resistant bacteria from animals to man has not been clearly documented, but this potential is supported by a variety of observations [74]: (1) animals fed antibiotics often have more antibiotic-resistant bacteria than those not fed antibiotics [75,76]; (2) antibiotic resistance genes can be transferred to foodborne pathogens [77,78,79]; and (3) farm workers sometimes carry more antibiotic-resistant bacteria than urban counterparts even though they are not receiving therapy [80,81]. Some studies indicated that antibiotic-resistant bacteria decreased after the use of antibiotic in animal feed was suspended [82,83], but there have been conflicting reports regarding this effect [10].

Traditional mechanisms of antibiotic resistance are not a good model for ruminal ionophore resistance. When Dawson and Boling [84] monitored the monensin sensitivity of bacteria from calves, they found that nearly 60% of the bacterial isolates were monensin-resistant prior to treatment, and monensin administration only caused a marginal increase in the resistance. The idea that ionophore resistance is a phenotypic selection rather than mutation or acquisition of foreign genes is further supported by measurements of monensin-dependent potassium efflux. When mixed ruminal bacteria were obtained from cattle consuming hay, the amount of monensin needed to catalyze half-maximal potassium depletion was approximately 0.2 μM, but this value increases eight-fold as soon as the cattle were supplemented with a daily dose of 350 mg monensin [85]. Because potassium efflux returned to its original value as soon as monensin was withdrawn (Fig. 4), it appeared that: (1) monensin resistance was prevalent before monensin is given; (2) increases in resistance occurred very rapidly; and (3) the increased resistance did not persist if the ionophore was not present.

Ionophores have routinely been fed to feedlot cattle for more than 25 years. However, there is little indication that the ionophore resistance of ruminal bacteria is increasing. In 1995, Mbanzamihigo et al. [86] noted that methane production of sheep was inhibited for as long as 28 days, and this decrease was still associated with an increase in propionate and a decrease in acetate-to-propionate ratio. Even more recently, Rogers et al. [87] fed monensin to sheep for 146 days. When monensin was withdrawn from the diet, there was an almost immediate decrease in acetate-to-propionate ratio.

In the 1970s, Chen and Wolin [30] selected monensin-resistant *P. ruminicola* cultures that were not resistant to lasalocid and vice versa, but other work indicates that ionophore-resistant bacteria are often resistant to other ionophores [5,88,89]. The susceptibility of ionophore-resistant ruminal bacteria to therapeutic antibiotics has not been extensively studied, but monensin-resistant *P. bryantii* and *C. aminophilum* cells were as sensitive to 22 commonly used antibiotics as their monensin-sensitive counterparts (Houlihan and Russell, unpublished results). In some cases, the monensin-resistant forms were actually more sensitive to these other antibiotics.

The idea that ionophore resistance of ruminal bacteria is not readily spread from one bacterium to another is supported by several recent studies. When Aarestrup [90] examined indicator bacteria (*Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium*), zoonotic bacteria (*Campylobacter*, *Salmonella*, and *Yersina*) and animal pathogens (*E. coli* and various staphylococci, and *Actinobacillus pleropneumoniae*) from Danish swine, cattle and poultry fed growth-promoting antimicrobials, monensin resistance was not frequently encountered. Furthermore, Butaye et al. [91] could not detect monensin resistance in 146 *E. faecium* and 166 *E. faecalis* strains isolated from farm and pet animals, and similar results were reported by Aarestrup et al. [92].

7. Potential impacts on human health

In the 1980s and 90s, the glycopeptide avoparcin was used as a growth promotant in pigs and chickens, and it was also marketed as an alternative to ionophores for cattle [93]. However, avoparcin is a vancomycin analog, and vancomycin is an important therapy for treating Gram-positive bacterial infections, particularly in AIDS patients. In 1995, Klare et al. [94] and Aarestrup et al. [95] isolated vancomycin-resistant enterococci from pigs and poultry fed avoparcin and suspicions of cross-resistance arose. Later research by Bager et al. [96] in Denmark confirmed a correlation between vancomycin-resistant *E. faecium* and avoparcin use. Nosocomial transmission has not been definitively demonstrated, but the European Union banned avoparcin use as a feed additive in 1997.

Some people have attempted to expand the analogy of avoparcin and vancomycin to ionophores [7], but this extrapolation is not well supported. Only some animals can be safely fed ionophores, and ionophores have never been (nor are likely to be) used as an antimicrobial for humans [19]. Because ionophores have a distinctly different mode of action from therapeutic antibiotics, there is little rationale for a common mechanism of resistance other than glycocalyx formation. Many ruminal bacteria are resistant to ionophores even if ionophores are not fed, and ionophore resistance seems to be a physiological selection

rather than a mutation or acquisition of foreign genes. Genes responsible for the ionophore resistance of ruminal bacteria have not been identified, and there is little evidence that ionophore resistance can be spread from one bacterium to another. Given these observations, use of ionophores in animal feed is not likely to have a significant impact on the transfer of antibiotic resistance from animals to man.

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