Antimicrobial Saponins of *Yucca schidigera* and the Implications of Their in Vitro Properties for Their in Vivo Impact

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The benefits of supplementing livestock diets with extracts from *Yucca schidigera* have been attributed to inhibition of selected gut microbes. The antimicrobial constituents were identified as three butanol-extractable 5β-spirostan-3β-ol saponins using *Bacillus pasteurii* and *Saccharomyces cerevisiae* as test prokaryotic and eukaryotic organisms, respectively. Although these saponins inhibited microbial growth at low cell densities, their impact was associated with adsorption to the microbes and they had no effect on dense microbial populations. The antimicrobial saponins were also observed to adsorb to the microbiota and other solids of the porcine cecum. The implications for gut microbes differ according to their ecological niche: Those sequestered to feed particles or the gut lining may accumulate saponins, whereas those flushed out synchronously with the digesta should be protected by high population densities.

Keywords: *Yucca schidigera; antimicrobial; saponin; animal feedstuff; supplements*

INTRODUCTION

The positive effects of dietary supplementation with 60−250 g tonne^{-1} *Yucca schidigera* extract on the growth rates (Anthony et al., 1994; Cline et al., 1996; Johnston et al., 1981; Mader and Brumm, 1987), feed efficiency (Johnston et al., 1982; Mader and Brumm, 1987), and health of livestock (Preston et al., 1985; Anthony et al., 1994; Balog et al., 1994) are well established. Until recently *Y. schidigera* extract was thought to be a potent in vitro inhibitor of the enzyme urease (urea amidohydrolase; EC 3.5.1.5) (Ellenberger et al., 1985; Asplund, 1991), and its in vivo properties were attributed to inhibition of gastrointestinal urease (Anthony et al., 1994; Balog et al., 1994; Sutton et al., 1992). However, the procedures used have since been shown to be flawed, and the previously determined inhibition of urease has since been shown to be almost completely artifactual (Killeen et al., 1994). The in vivo inhibition of gut urease activity by *Y. schidigera* supplements now appears untenable on the basis of studies in vitro (Killeen et al., 1994) and in vivo (Killeen et al., 1998). Other proposed mechanisms of action include stimulation of microfloral growth (Peestock, 1979), direct binding of ammonia (Headon et al., 1991), and inhibition of selected gut microbes (Hussain and Cheeke, 1995), rumen protozoa in particular (Wallace et al., 1994). *Y. schidigera* clearly influences microbial processes in a variety of fermentation systems (Balog et al., 1994; Killeen et al., 1998; Preston et al., 1987; Sutton et al., 1992; Gibson et al., 1985; Ellenberger et al., 1985; Grobner et al., 1982; Goodall and Matsushima, 1979; Hussain and Cheeke, 1995; van Nevel and Demeyer, 1990; Peestock, 1979; Goodall et al., 1979), and it has been suggested that this may be the result of its antimicrobial activity in vitro (Wallace et al., 1994; Hussain and Cheeke, 1995).

The following study was therefore conducted to identify the active antimicrobial constituents of *Y. schidigera* extract and to study the factors affecting its potency. A number of common gut bacteria, including *Prevotella (Bacteroides) ruminicola*, *Streptococcus bovis* (Wallace et al., 1994), *Lactobacillus plantarum*, *Bacteroides vulgatus* (K. E. Newman, personal communication), *Butyrivibrio fibrisolvens* (Wallace et al., 1994; K. E. Newman, personal communication), and rumen protozoa (Wallace et al., 1994), are inhibited by *Y. schidigera*

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extracts in vitro. However, we chose to use *Saccharomyces cerevisiae* and *Bacillus pasteurii* as test eukaryotic and prokaryotic organisms, respectively, because these are inhibited by lower concentrations of the extract than the gut microbes tested thus far (Wallace et al., 1994; K. E. Newman, personal communication). The use of such sensitive test organisms allows the study of factors influencing the antimicrobial potency of *Y. schidigera* extracts at levels of microbe susceptibility lower than those found so far but which may yet be identified in gut microbes. These organisms are also readily amenable to the agar-disk diffusion method described herein, which allows quantification of susceptibility and antimicrobial activity as a continuous variable rather than the incremental series of breakpoints characteristic of more traditional methods (Delignette-Muller and Flandrois, 1994). Furthermore, their high susceptibility to inhibition by *Y. schidigera* allows such assays to be conducted at the very low extract concentrations similar to typical feed inclusions.

MATERIALS AND METHODS

Materials. The same 22.6% (w/v) solids commercial liquid *Y. schidigera* concentrate (DeOdorase) as used in previous mechanistic work (Killeen et al., 1994) was used for this study. The concentrate was extracted into *n*-butanol, and both fractions were dried in vacuo, yielding viscous residues that retained traces of butanol. The residues were resuspended in H_2O overnight and lyophilized to yield dry resins, which were dissolved and made up to their original volume in H_2O.

Maintenance of Microbes. *B. pasteurii* (NCIMB 8841) was maintained on 2% (w/v) tryptone, 1% (w/v) yeast extract, and 2% (w/v) NH_4Cl, adjusted to pH 9.0 with NaOH, and autoclaved at 105 °C for 30 min. *S. cerevisiae* (NCYC 1026) and *Escherichia coli* JM-109 (Yanisch-Perron et al., 1985) were maintained on YPD and LB broths (Difco), respectively.

Detection and Quantification of Antimicrobial Activity against *B. pasteurii* and *S. cerevisiae*. *Growth Inhibition Studies in Liquid Culture.* Studies of the potency of *Y. schidigera* extract as well as its fractions and purified saponins were carried out in the maintenance broths described above. Aliquots (20 mL) of broth were mixed with 50−200 μL aliquots of samples that had been made up and diluted to a range of concentrations in *n*-butanol/ethanol/H_2O (1:1:1). Solvent-dissolved samples were used because of their sterility, ease of handling without foaming, and the insolubility of partially purified saponins in water. The volume of solvent-dissolved sample was varied among experiments according to the potency of the sample. However, at the upper end of this sample size range, a very slight lengthening of the lag time by the solvent mixture alone was sometimes observed. Therefore, within an experiment, the volume of solvent was kept constant, including a negative control in which solvent without solutes was added. The treated media were then inoculated with 1% (v/v) of stationary phase cultures of *B. pasteurii* or *S. cerevisiae*, containing approximately 10^8 CFU mL^{-1} as counted on the agar media described above. Subsequent growth was monitored as optical density at 600 nm (OD_{600nm}).

Agar Plate Diffusion Assay. We chose to adapt the agar-disk diffusion technique and regression analysis previously described (Delignette-Muller and Flandrois, 1994) because it allows the expression of microbial susceptibility as a continuous variable rather than an incremental one, as is typical of more traditional agar or broth dilution and disk diffusion methods. Such methods are based on assumed identity of the minimum inhibitory concentration (MIC) as determined by commonly used methods and the inhibitory concentration in diffusion (ICD), which represents the antibiotic concentration in the agar at the threshold of an inhibition zone (Kronvall and Ringertz, 1991; Delignette-Muller and Flandrois, 1994). This identity has been experimentally demonstrated for a number of antimicrobial agents, tested against an array of bacterial species and strains (Chapuis et al., 1996; Guerin-Faublee et al., 1996; Delignette-Muller and Flandrois, 1994). As described below, the ICD is calculated by regression analysis of the relationship between the size of the zone of inhibition and the amount of sample applied.

At least three aliquots of different volumes of the sample in solvent or water were dried under a warm air stream onto disks of blotting paper (6.3 mm diameter). The appropriate agar media [liquid media above plus 1.5% (w/v) agar] were cooled to 50 °C and inoculated to 0.5% (v/v) with stationary phase cultures of *B. pasteurii* or *S. cerevisiae*, and 100 mL was immediately poured into sterile 22.7 × 22.7 cm plates. The disks were placed on the surface of the agar plates after they had solidified and cooled to room temperature. The plates were inverted and incubated at 4 °C for 4 days to allow for the slow diffusion of the saponin micelles. They were then transferred to 30 °C for 2 days to allow growth of the test microbe and hence visualization of the zones of inhibition. Two orthogonal diameters of the resulting clearance zones were then measured with a Vernier callipers. Note that although the mixed micelle nature of saponin solutions slows diffusion and hence analysis, it also allows the measurement of microbe inhibition in such complex mixtures because they codiffuse. If they diffused independently, the analysis would measure only the ICD of the component with the combination of potency and diffusion rate that produced the largest rings.

The ICD was calculated, as previously described (Delignette-Muller and Flandrois, 1994), from the slope and intercept yielded by regression of the radius of the clearance zone (r) and the concentration of sample in the area under the disk at time zero (C_0)

$$\mathrm{ICD} = 2\alpha \exp(\beta) \qquad (1)$$

where

$$\ln C_0 = \alpha r^2 + \beta \qquad (2)$$

The term C_0 was calculated by dividing the weight (solids) or volume (liquids) of sample applied (Q) by the volume of the agar cylinder under the disk (V_{disk}).

$$C_0 = Q/V_{disk} \qquad (3)$$

V_{disk} is usually calculated from the depth of agar and the radius of the paper disk (Delignette-Muller and Flandrois, 1994). In this study, however, very thin agar plates were used to enhance sensitivity, so the depth of the agar could not be measured directly with sufficient precision. Assuming uniform depth throughout the plate (undistorted zones of clearance were consistent with this assumption), the fraction of the total plate volume covered by the disk is equal to the fraction of its area covered by the disk. Thus, the volume under the disk was calculated from its area (A_{disk}), the area of the plate (A_{plate}), and the total volume of agar poured into it (V_{plate}).

$$V_{disk} = V_{plate} A_{disk}/A_{plate} \qquad (4)$$

In all experiments, radii from at least three disks, containing different amounts of sample, were used in the regression analysis of that replicate. The measurement of ICD using three disks has been shown to be more precise than MIC determination by agar dilution methods (Delignette-Muller and Flandrois, 1994).

To compare the total antimicrobial activity of various *Y. schidigera* fractions, the capacity of a fraction to inhibit growth of a microorganism was defined as its maximum inhibitory volume (MIV), that is, the volume to which it would have to be diluted to reach its ICD for that organism. The MIV was calculated by dividing the weight of the fraction by its ICD and allows the comparison of the yield recovered in fractions of different purities and concentrations.

Saponin Identification by Thin-Layer Chromatography (TLC). Plates (0.2 mm depth glass backed Kieselgel 60)

were developed once in octan-2-ol/3-methylbutanol/acetone/methanol/water (3:5:2:1:1), dried at 120 °C, stained with 10% H_2SO_4, and heated (120 °C 20 min.). Plates were sometimes also stained selectively for saponins using vanillin (Oakenfull, 1981).

Purification of Antimicrobial Constituents. All fractions of *Y. schidigera* extract yielded by the following purification were screened for antimicrobial activity using *S. cerevisiae* and *B. pasteurii* by the agar diffusion technique. Aqueous *Y. schidigera* extract (1 L) was extracted into 2 × 1 L of *n*-butanol and dried in vacuo. The extract was loaded onto a dimethylsilyl silica column in 2:3 methanol/water and eluted sequentially with 2:3, 1:1, and 7:3 methanol/water, followed by methanol. This yielded two distinct saponin fractions, groups A and B, as identified by vanillin-stained TLC, in the 1:1 and 7:3 eluates. As it was found to contain the bulk of the antimicrobial activity, the group B fraction was loaded onto a normal-phase silica column in $CHCl_3$/acetone (4:1) and washed with $CHCl_3$/acetone (4:1) and then a stepwise gradient of $CHCl_3$/acetone/methanol/H_2O (74:20:10:1−49:20:10:1). The three main antimicrobial constituents, B1, B2, and B3, were eluted in that order.

Spectroscopic Studies. IR and UV spectra were recorded on Perkin-Elmer 1600 series and Shimadzu UV-160A spectrophotometers, respectively. ^1H and ^{13}C NMR spectra, including homonuclear (COSY) and heteronuclear correlation (HETCOR), and distortionless enhancement by polarization transfer (DEPT) experiments were recorded at 270 and 68 MHz, respectively, on a JEOL JNM-GX270 FT-NMR spectrometer and at 400 and 100 MHz, respectively, on a Bruker 400 FT-NMR spectrometer. All chemical shift (δ) values reported are in parts per million, relative to tetramethylsilane (TMS), which was included as an internal standard. The labile hydroxyl protons of intact saponins were exchanged for deuterium (D) by stirring in *n*-butanol/D_2O (1:2). Fast atom bombardment mass spectroscopy (FAB-MS) spectra, detected in positive ion mode, of samples in nitrobenzyl alchohol (NBA) matrixes were recorded on a Kratos MS80 RFAQ hybrid mass spectrometer.

***B1*:** off-white amorphous powder; IR ν_{max} (KBr) 3350 (O−H stretch), (2900 C−H stretch) 1645 (C=C stretch), 1450, 1380, 1245, 1153, 1076, 1045 (spiroketal) 918 (25S spiroketal), 895 (25R spiroketal), 851 (spiroketal), 602 cm^{-1}; FAB-MS (*e/z*) 763 $[H + M]^+$, 579 $[H + spirostanol + glc]^+$, 417 $[H + spirostanol]^+$, 399 $[H + spirostanol - H_2O]^+$, 381 $[H + spirostanol - 2H_2O]^+$, 363 $[H + spirostanol - 3H_2O]^+$, 347, 329, 325, 317, 307, 185.

***B2*:** creamy amorphous powder; IR ν_{max} (KBr) 3350 (O−H stretch), (2900 C−H stretch) 1644 (C=C stretch), 1451, 1378, 1242, 1157, 1078, 1044 (spiroketal) 918 (25S spiroketal), 897 (25R spiroketal), 851 (spiroketal),754, 601 cm^{-1}; FAB-MS (*e/z*) 895 $[H + M]^+$, 763 $[H + M - xyl]^+$, 497, 495, 479, 417 $[H + spirostanol]^+$, 399 $[H + spirostanol - H_2O]^+$, 381 $[H + spirostanol - 2H_2O]^+$, 363 $[H + spirostanol - 3H_2O]^+$, 347, 325, 317, 185.

***B3*:** creamy amorphous powder; IR ν_{max} (KBr) 3350 (O−H stretch), (2900 C−H stretch) 1641 (C=C stretch), 1451, 1378, 1229, 1157, 1078, 1045 (spiroketal) 918 (25S spiroketal), 898 (25R spiroketal), 850 (spiroketal), 597 cm^{-1}; FAB-MS (*e/z*) 895 $[H + M]^+$, 763 $[H + M - xyl]^+$, 497, 495, 479, 417 $[H + spirostanol]^+$, 399 $[H + spirostanol - H_2O]^+$, 381 $[H + spirostanol - 2H_2O]^+$, 363 $[H + spirostanol - 3H_2O]^+$, 347, 325, 317, 185.

Degradative Studies. Each of the three saponins **B1**−**B3** (50 mg) were hydrolyzed in 10 mL of *n*-butanol/acetone/ethanol/water/HCl (2:1:5:5:1) at 90 °C for 3 h, and the aglycon was then extracted with diethyl ether (Kaneda et al., 1987). Sapogenins yielded by degradative studies were identified by TLC (Kaneda et al., 1987) and gas chromatography (GC) (Killeen et al., 1998). Authentic standards of 5β-spirostan-3β-ol, 5β-spirostan-2β,3β-diol, and 5β-spirostan-2α,3β-diol, isolated from *Y. schidigera* (Kaneda et al., 1987), were kindly provided by Dr. N. Kaneda (Eli Lilly Research, Tokyo). Solvents were removed from the remaining acid phase with a stream of N_2 at 50 °C. The aqueous hydrolysates were then neutralized with an excess of Ag_2CO_3 (∼1.7 g), filtered through sintered glass, centrifuged to remove AgCl and excess Ag_2CO_3, and then lyophilized. Sugars in these aqueous hydrolysate fractions were identified by TLC (Menzies et al., 1978) with mannose, glucose, fucose, xylose, rhamnose, galactose, and arabinose standards.

Treatment of *Y. schidigera* Extract with Microbial Suspensions. *Axenic Cultures.* Aliquots (0, 10, and 50 mL) of stationary phase 2 L cultures of all three microbes were washed by centrifuging (30 min × 3000*g*) and resuspending four times in 50 mL of cold diluent [1% NH_4Cl adjusted to pH 9.0 with NaOH for *B. pasteurii*, 80 mM KH_2PO_4 (pH unadjusted) for *S. cerevisiae* and one-fourth strength Ringer solution for *E. coli*]. This resulted in suspensions containing 0, 0.2, and 1.0 times the original microbial density of the original culture, respectively.

Porcine Cecal Digesta. The ceca of two Large White pigs (one of each sex) were ligated and excised and their combined contents (397 g) mixed with 552 mL of diluent under anaerobic conditions (20% H_2 in CO_2) (Bryant and Burkey, 1953). The mixture was divided into triplicates, chilled on ice, and then washed by centrifuging and resuspending five times in cold anaerobic diluent (Bryant and Burkey, 1953). Equivalent volumes of the diluent were prepared as controls.

Incubation of Extract with Microbe/Digesta Suspensions. Triplicates of the last resuspensions of the axenic cultures and digesta solids and their controls included 500 and 200 mg L^{-1} *Y. schidigera* extract, respectively. These mixtures and their triplicate negative controls (no *Y. schidigera*) were shaken at 200 rpm and 30 °C (*B. pasteurii* and *S. cerevisiae*) or 37 °C (*E. coli* and digesta solids) for 1 h (under H_2-CO_2 for the digesta) and then centrifuged again.

Analysis of Antimicrobial Activity, Saponins, and Sapogenins. The supernatant of the axenic cultures (40 mL) and digesta solids (45 mL) were extracted exhaustively with *n*-butanol. In the case of the butanol extracts from the digesta, which contained a dark residue from the buffer/butanol interface, the extract was filtered through a cotton wool plug in a Pasteur pipet. The residue in the plug was washed with *n*-butanol, and the washings were added to the original filtrate. Extracts from the control treatment with no digesta were treated identically. These butanol extracts were dried in vacuo and redissolved in 200 μL of *n*-butanol/ethanol/water (1:1:1) for analysis of antimicrobial activity and, in the case of the axenic culture treatments, saponin and total sapogenin content.

Saponins were analyzed by TLC as described above. Total sapogenin content was quantified by reaction with anisaldehyde: the redissolved butanol extracts were diluted in methanol/water (1:1) and assayed, using diosgenin as a standard, as previously described (Hiai and Wu, 1976) except that 1% (v/v) anisaldehyde in ethanol was used instead of vanillin because this greatly enhances the specificity, sensitivity, and precision of the method (Baccou et al., 1977). Absorbance was measured at 430 nm.

The antimicrobial activity of the extracted and redissolved saponins was quantified by the agar-disk diffusion method. When antimicrobial activity in this experiment was quantified, *S. cerevisiae* was always used as the test microbe because of its high susceptibility, which allowed the detection and quantification of activity from <1 mg of extract. The effects of microbes/digesta on the antimicrobial activity of *Y. schidigera* were expressed by calculating the observed ICD (ICD_{obs}) of the treated solution, based on the ICD of the reconstituted extract and the concentration factor of its extraction and dissolution in a smaller volume. The ICD_{obs} represents the relative concentration required to negate the antimicrobial activity of the sample under the particular experimental conditions described. Thus, ICD values of >1 represent solutions incapable of inhibiting the test organism without the solvent extraction and concentration step. The ICD_{obs} is a concentration factor and is therefore dimensionless and has no units.

Statistics. All variances are presented as the standard error of the mean (SEM). In all of the experiments in which antimicrobial activity was determined by the disk diffusion

Table 1. Potencies and Total Antimicrobial Activities of *Y. schidigera* and Saponin Fractions Thereof, As Tested against *B. pasteurii* and *S. cerevisiae*[a]

		ICD (mg L^{-1})		MIV (L)		yield (%)	
fraction	amount (mg)	*B. pasteurii*	*S. cerevisiae*	*B. pasteurii*	*S. cerevisiae*	*B. pasteurii*	*S. cerevisiae*
crude extract	1000000	427 ± 41	177 ± 24	2341	5650	100	100
group A	34710	225 ± 32	114 ± 23	154	305	6.6	5.4
group B	21920	37.6 ± 5.8	4.47 ± 1.53	583	4904	24.9	86.8
B1	290	ND[b]	0.65 ± 0.075	ND[b]	446	ND[b]	7.9
B2	641	7.60 ± 0.065	2.07 ± 0.451	84	310	3.6	5.5
B3	153	8.65 ± 1.03	2.23 ± 0.806	18	69	0.8	1.2

[a] Values are means of at least three replicates and regressions ± SEM as determined by the agar diffusion method. [b] Could not be measured, probably due to the low solubility and slow diffusion properties that were observed in the *S. cerevisiae* measurements. Dilutions in liquid media showed it to have an MIC of 10−20 mg L^{-1}.

method, each replicate yielded sufficient sample for at least three disks, and the results from each replicate were separately analyzed by regression. The variances presented are therefore those between replicates after summarization of all the data from that replicate into a single ICD value.

RESULTS AND DISCUSSION

Antimicrobial Activity of *Y. schidigera* Extracts. Preliminary liquid culture and agar plate assays showed that the growth of both *S. cerevisiae* and *B. pasteurii* but not *E. coli* were inhibited by *Y. schidigera* extracts. The nature of the MIC assay in liquid culture dictates that the true MIC lies between the observed MIC and the next lowest concentration of *Y. schidigera* and is thus best expressed as the range or mean of these two breakpoints (Delignette-Muller and Flandrois, 1994). The MIC ranges observed in this way for *S. cerevisiae* and *B. pasteurii* were 200−400 and 400−600 mg L^{-1} respectively. The agar plate method was found to be extremely sensitive (Figure 1a) and readily applicable to regression analysis as described under Materials and Methods (Figure 1b). The ICD values of crude *Y. schidigera* determined in this way for *S. cerevisiae* and *B. pasteurii* (Table 1) compare well with these MIC ranges, as predicted by their theoretical identity (Kronvall and Ringertz, 1991; Delignette-Muller and Flandrois, 1994) and experimentally demonstrated correlation (Delignette-Muller and Flandrois, 1994; Guerin-Faublee et al., 1996; Chapuis et al., 1996). On this basis, the two former microbes are at least as sensitive to *Y. schidigera* inhibition as any eukaryotes and prokaryotes studied so far (Wallace et al., 1994; K. E. Newman, personal communication). Because of its sensitivity to *Y. schidigera* activity, *S. cerevisiae* was chosen as the test organism for all subsequent quantifications of antimicrobial activity of *Y. schidigera* in mechanistic studies by the agar-disk diffusion method.

Identification of Antimicrobial Saponins. This activity was found to be entirely butanol-extractable, as has been reported for its antiprotozoal activity (Wallace et al., 1994), and consistent with the known properties of monodesmoside saponins (Price et al., 1987). The butanol extract was found to contain two main saponin fractions by reverse phase column chromatography (Figure 2a), which were the only fractions with detectable antimicrobial activity. The group B saponin fraction was found to contain most of the antimicrobial activity, and its three main antimicrobial constituents were separated by normal phase column chromatography (Figure 2b; Table 1). These antimicrobial saponins were also identified in aqueous and methanolic extracts of an authentic *Y. schidigera* specimen (voucher deposited at Rancho Santa Ana Botanic Garden, 1500 N. College Ave., Claremont, CA 91711).

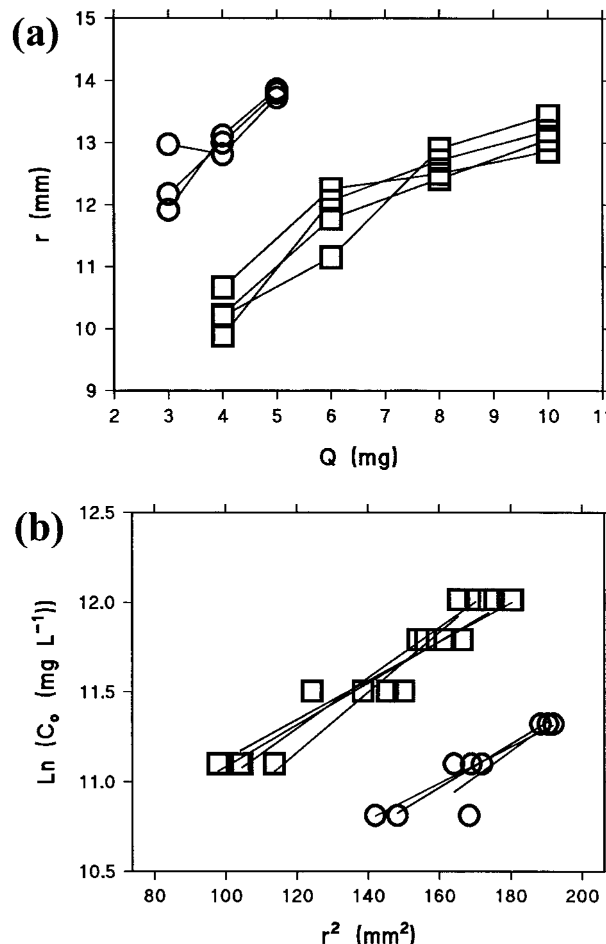

Figure 1. Detection and measurement of *Y. schidigera* antimicrobial activity against *B. pasteurii* (□) and *S. cerevisiae* (○) using the agar plate diffusion method: (a) radius (*r*) of the rings produced by various quantities of crude extract (*Q*) loaded onto each disk; (b) regressions of these transformed values used to calculate ICD (see Materials and Methods).

Partially purified compounds **B1**, **B2**, and **B3** all inhibited both *B. pasteurii* and *S. cerevisiae* at low concentrations (Table 1).

All three compounds exhibited peaks at *m*/*z* 363, 381, 399, and 417 in their FAB-MS spectra, consistent with their previously suggested steroidal nature (Hussain and Cheeke, 1995; Wallace et al., 1994). The aglycons of all three compounds yielded by acid hydrolysis were identified as 5β-spirostan-3β-ol by TLC and GC. Carbon-13 NMR of the aglycons (Table 2) showed them to be a mixture of the C-25 epimers of 5β-spirostan-3β-ol, sarsasapogenin (25S), and smilagenin (25R). The presence of similar peaks, consistent with the presence of the two diastereomers, was also noted in the ^{13}C NMR

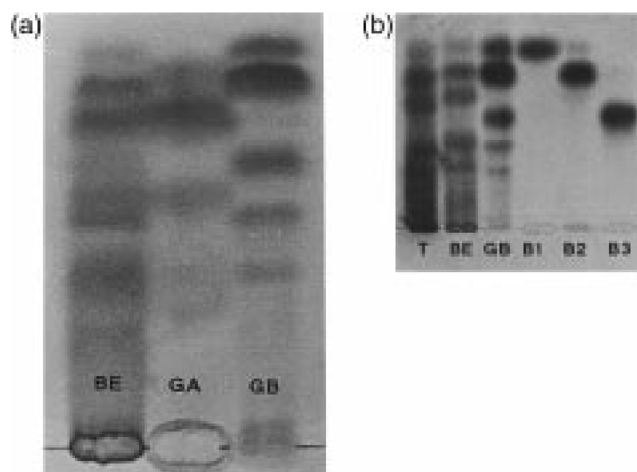

Figure 2. TLC profiles of *Y. schidigera*, its major antimicrobial fractions, and their purified active constituents: (a) visualized by anisaldehyde staining (BE, butanol extract; GA, group A; GB, group B) (all equivalent to 10 mg of *Y. schidigera*); (b) visualized by H_2SO_4 staining for organic compounds [T, total *Y. schidigera* extract (1 μL); BE, butanol extract (equivalent to 1 μL of T); GB, group B (equivalent to 1 μL of T); **B1**, **B2**, and **B3** (50 μg each)].

Table 2. ^{13}C NMR Identification of the Aglycons of Compounds B1, B2, and B3

observed δ			assignment		
B1	**B2**	**B3**	C no.	diastereomer	literature δ (Agrawal et al., 1985)
14.4	14.3	14.4	21	*S*	14.3
14.6	14.6	14.5	21	*R*	14.5
16.0	16.1	16.0	27	*S*	16.1
16.5	16.5	16.5	18	*RS*	16.4
17.1	16.9	17.0	27	*R*	17.1
20.9	21.0	21.0	11	*RS*	20.9
23.9	23.9	24.0	19	*RS*	23.9
25.7	25.7	25.7	24	*S*	25.8
25.9	26.0	25.9	25	*S*	26.0
26.6	26.5	26.6	6, 7	*RS*, *RS*	26.6
27.0	27.3	27.1	23	*S*	27.1
27.8	27.9	27.8	2	*RS*	27.8
28.8	29.0	28.9	24	*R*	28.8
30.0	30.0	30.0	1	*RS*	29.9
30.3	30.3	30.2	25	*R*	30.3
31.3	31.4	31.5	23	*R*	31.4
31.8	31.8	31.7	15	*R*	31.7
33.5	33.5	33.6	4	*RS*	33.5
35.3	35.4	35.3	8, 10	*RS*, *RS*	35.3, 35.3
36.5	36.7	36.5	5	*RS*	36.6
39.9	40.0	40.1	12	*RS*	40.0
40.3	40.2	40.3	9	*RS*	40.3
40.7	40.8	40.7	13	*RS*	40.7
41.6	41.8	41.7	15	*S*	41.7
42.1	42.2	42.1	20	*S*	42.2
56.5	56.5	56.5	14	*RS*	56.5
62.1	62.1	62.3	17	*R*[a]	62.3
62.3	62.4	62.4	17	*S*[a]	62.3
65.2	65.3	65.3	26	*S*	65.2
66.9	66.9	66.7	26	*R*	66.8
67.1	67.0	67.0	3	*RS*	66.9
80.9	80.9	80.8	16	*S*[b]	80.9
81.0	81.0	80.9	16	*R*[b]	80.9
109.3	109.3	109.2	22	*R*	109.2
109.8	109.8	109.8	22	*S*	109.7

[a,b] Assignments with common superscripts are interchangeable

spectra of the intact compounds. Furthermore, the IR spectra of all three intact compounds displayed absorption peaks at both 918 and 895−898 cm^{-1}, confirming the native saponins to be 25R and 25S epimeric mixtures (Wall et al., 1952).

TLC of the aqueous fraction yielded by acid hydrolysis of **B1**, **B2**, and **B3** showed them all to contain glucose. The FAB-MS spectra of all three compounds contained a peak *m*/*z* of 579, indicating that the glucose was linked directly to carbon three of the 5β-spirostan-3β-ol. Singlet peaks consistent with an alkene carbon were found at ~145 ppm in the ^{13}C NMR spectra of all three compounds. Although the presence of an alkene contaminant cannot be ruled out, strong peaks at ~1643 cm^{-1} were present in the IR spectra of all three compounds, indicating this group is probably a part of the saponins per se. FAB-MS peaks corresponding to this alkene fragment, with and without glucose attached, indicate that in all three compounds the glucose is linked directly to this unsaturated substituent. Xylose was identified as a substituent of **B2** by TLC of its hydrolysate and from the peak at *m*/*z* 763 in its FAB-MS spectrum, which corresponds to the ejection of xylose. Similarly, galactose was identified by TLC in the aqueous hydrolysate of **B3**, and the same FAB-MS peak corresponding to the ejection of the galactose was found.

^{1}H and ^{13}C NMR and elemental analysis of these compounds showed impurities to be present, which confounded unambiguous structural assignment by ^{1}H−^{1}H and ^{13}C−^{1}H correlation as well as DEPT experiments. Furthermore, attempts to purify these compounds to homogeneity by reverse phase high-performance liquid chromatography (RP-HPLC) were frustrated by the problematic solubility properties of these saponins: Although readily soluble in water and short-chain alchohols in their crude form, the compounds yielded by normal phase column chromatography could only be dissolved to useful concentrations in relatively complex solvent mixtures. The insolubility of these compounds, once partially purified, is reflected in the complex solvent mixtures necessary for the TLC, normal phase CC, and degradative studies. These low-polarity mixtures containing *n*-butanol, chloroform, or pyridine did not allow further purification by RP-HPLC.

Dependence of the Inhibitory Properties of *Y. schidigera* on Microbial Population Density. The inhibition of *B. pasteurii* and *S. cerevisiae* by *Y. schidigera* extract was negated by allowing the growth of cultures to high densities, similar to those of microbial populations found in vivo (Figure 3). The threshold concentration at which most cytotoxic and antimicrobial surfactants, including saponins, induce cytolysis pertains to their concentration in the membranes/microbes themselves rather than their overall concentration in bulk solution (Schulman et al., 1955). Therefore, their antimicrobial potency is inversely related to the biomass and cell density of the target organism; at higher cell densities each cell carries a lower saponin load, which may not exceed the threshold required for the formation of the porous two-dimensional micelles responsible for toxicity and inhibition (Schulman et al., 1955). The lack of observed antimicrobial activity by allowing culture growth was probably due to increased microbial numbers. However, the possibility of changes in the metabolic state of the microbes or the makeup of their surrounding medium could not be discounted on the basis of this experiment alone.

Dilutions (500 mg L^{-1}) of the extract were therefore treated with various quantities of all three microbes, which were added as washed suspensions of stationary phase cultures. The cells were removed, and the free

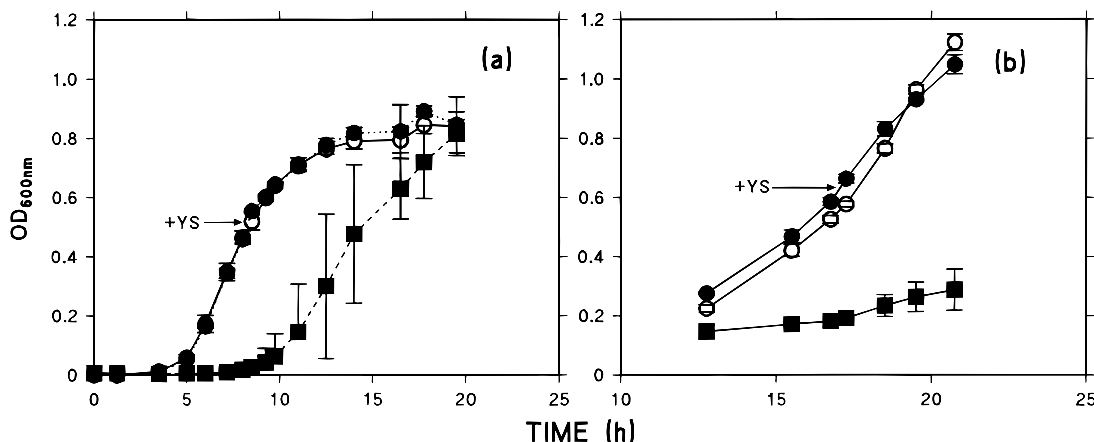

Figure 3. Influence of target organism culture density on the antimicrobial potency of *Y. schidigera* extracts as determined with (a) *B. pasteurii* (1000 mg L^{-1} *Y. schidigera*) and (b) *S. cerevisiae* (500 mg L^{-1}): (○) control (no *Y. schidigera*); (■) *Y. schidigera* added after autoclaving but before inoculation; (●) *Y. schidigera* added after inoculation and growth at the point indicated by the arrow. Each point represents the mean ± SEM of triplicate cultures.

antimicrobial activity remaining in the extracellular medium was quantified. The presence of microbial cells reduced free antimicrobial activity levels, and a clear dependence of free antimicrobial activity on cell density was observed (Figure 4a). This free antimicrobial activity reflects the concentration of saponins to which single cells in the population are exposed. Furthermore, the observed increase in the ICD_{obs} reflects the alleviation of the saponin burden on individual cells by their sheer numbers (Figure 4a). The disappearance of activity correlated directly to the susceptibility of the organism used to treat the extract dilution (Figure 4a). Although a less dramatic effect on total spirostanol content was observed (Figure 4b), TLC analysis showed that treatment with *B. pasteurii* and particularly *S. cerevisiae* selectively removed B1 and B2 but not the other numerous saponins present (Figure 4c). Note the comigration of saponin **B2** with one of the group A saponins (Figure 2a); although not visible in monochrome, the remaining bands at this point in the 1.0 × *B. pasteurii* and *S. cerevisiae* treatments (Figure 4c) are almost exclusively composed of the group A constituent, which stains green rather than black with H_2SO_4 charring, as is characteristic of **B2**. Thus, most of **B1** and **B2**, the most potent antimicrobial constituents of the extract, are removed, presumably adsorbed, by the cells, leaving the other, relatively impotent, saponins free in solution.

The correlation between the ability of *Y. schidigera* saponins to adsorb to microbial cells and their inhibitory potency may seem intuitive, but many surfactants can exert their effects in free solution by simply altering the surface tension of the extracellular medium (Schulman et al., 1955). However, most saponins are only weakly inhibitory to prokaryotes such as *B. pasteurii* because, unlike *S. cerevisiae*, which is eukaryotic, they lack membrane sterols with which the saponins complex to disrupt membrane function (Price et al., 1987; Schulman et al., 1955). There is no clear indication as to why *B. pasteurii* is inhibited (Table 1), but up to 1600 mg L^{-1} of *Y. schidigera* extract has no impact on *E. coli*, *Ruminococcus albus*, *Streptococcus bovis*, *Fibrobacter succinogenes*, *Selenomonas ruminatum*, *Enterococcus faecium*, *Staphylococcus aureus*, *Pediococcus acidilactici*, *Lactobacillus pentosus*, and even *Bacillus* species such as *B. subtilis* and *B. megaterium* (K. E. Newman, personal communication). Presumably their cell membrane structures and constituents are critical determinants of saponin adsorption and toxicity.

Effect of Gut Digesta on Antimicrobial Properties. To determine whether the microbial population of the hindgut had sufficient adsorptive capacity to similarly attenuate the antimicrobial activity of extract concentrations approximating those used in feed, dilutions of *Y. schidigera* extract (200 mg L^{-1}) were treated with washed suspensions of cecal digesta from pigs. As for the suspensions of axenic microbe cultures described above, the cells and other solids were removed and free antimicrobial activity remaining in solution was quantified. Of three triplicate digesta treatments, only one had detectable antimicrobial activity ($ICD_{obs} = 5.1$), whereas the controls (no digesta) were readily detected and quantified ($ICD_{obs} = 2.24 \pm 0.068$). Thus, gut microbes and digesta particles have the same ability to adsorb antimicrobial saponins as suspensions of single microbes (Figure 4). Presumably, the impact of *Y. schidigera* on the growth of gut microorganisms in vivo is therefore dependent on population density in the same way that it is for single microbes in vitro (Figure 3). It is worthwhile to consider that all three treatments with gut solids resulted in recoverable antimicrobial activity which was at least 5-fold too weak to inhibit even the highly susceptible test organism.

Unfortunately, this more representative experiment cannot be interpreted quite as unambiguously as the equivalent experiment with suspensions of single microbes. Unlike the treatments with axenic cultures in which the microbial population is essentially homogeneous, differences in the affinities or adsorption rates of saponins to different members of the population cannot be distinguished. Also, neither activation of otherwise inert components by digestive processes nor direct effects on sparser populations such as the microflora of the small intestine should be disregarded. Furthermore, these experiments model only the gut lumen where microbes and digesta pass through synchronously, and the implications for other microbes may be quite different. The adsorption of saponins to microbes such as ciliate protozoa, which are retained in the rumen or hindgut, may result in their accumulation and render these populations more prone the inhibitory effects of *Y. schidigera*.

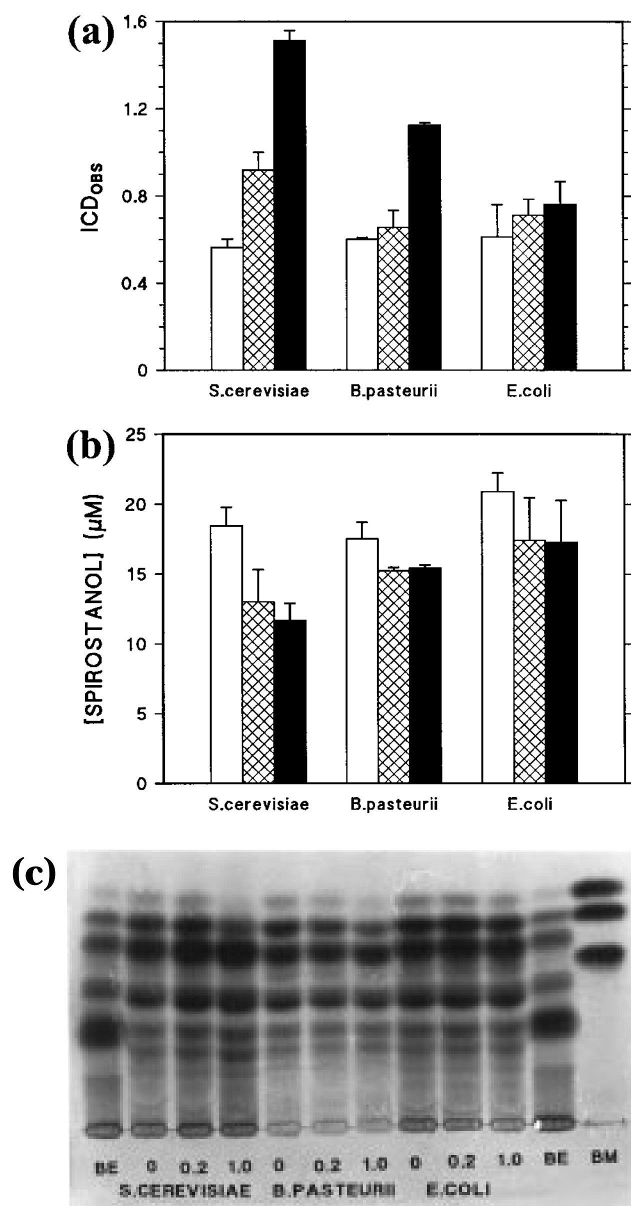

Figure 4. Influence of treatment of triplicate 500 mg L^{-1} dilutions of *Y. schidigera* extract with washed microbe suspensions at 0 (open bars), 0.2 (hatched bars), and 1.0 (solid bars) times the density of stationary phase cultures on (a) antimicrobial activity against *S. cerevisiae*, expressed as ICD_{obs}, (b) butanol-extractable spirostanol, and (c) H_2SO_4-stained TLC saponin profile (pooled triplicates) equivalent to 1.5 mg of *Y. schidigera* extract (BE, butanol extract equivalent to 1.5 mg of *Y. schidigera* extract; BM, mixture of 15 μg each of **B1**, **B2**, and **B3**). For (a) and (b) all error bars represent the SEM.

OVERVIEW

Although it is abundantly clear that *Y. schidigera* can influence microbial populations, it is difficult to resolve unambiguously direct from indirect mechanisms of microbe inhibition and/or stimulation, either in vivo or in mixed cultures in vitro. If, for example, numbers of certain microbes were augmented or diminished, one could not be sure that the effect was not caused indirectly via more direct effects on the growth and/or metabolism of other organisms in the microbial milieu or even on the host. Inhibition of selected population subsets in vivo cannot be totally discounted on the basis of these experiments. However, the considerable attenuation of *Y. schidigera* antimicrobial activity by the sheer density of gut populations is surely of considerable significance. In addition to adsorptive dilution by the microbiota, attenuation of the antimicrobial activity may also be caused by complexation with minerals and other steroids in the digesta as occurs with other saponins (Milgate and Roberts, 1995; Price et al., 1987). Thus, MIC values of *Y. schidigera* against such microbes, determined by inoculation of batch cultures with small aliquots of microbes, grossly overestimate the antimicrobial potency of *Y. schidigera* in practical situations. Furthermore, the MIC values for a wide range of gut and nongut microbes are higher than typical feed inclusions (Wallace et al.,1994; K. E. Newman, personal communication). Although high concentrations (1000–10000 mg L^{-1}) of *Y. schidigera* inhibit the growth of *S. bovis* and *B. fibrisolvens* and reduce the bacteriolytic activity of rumen protozoa in vitro (Wallace et al., 1994), none of these effects are observed at supplementation levels approaching those commonly applied to livestock feeds (60–250 mg kg^{-1}). Also, during digestion, feed becomes considerably diluted in the digestive secretions of the animal. For example, it is diluted ~2-fold as it enters the porcine caecum (Pfeiffer et al., 1995).

Rumen protozoa deserve special attention because of their critical role in rumen nitrogen metabolism (Jouany et al., 1988) and their inherent vulnerability to surfactants (Burggraaf and Leng, 1980). The implications of the adsorptive properties of *Y. schidigera* saponins for ciliate protozoa are more complex than for microbes, which are continuously growing and being flushed out of the gut in synchrony with the digesta: Rumen protozoa are retained in the gut by sequestration and may therefore accumulate saponins passing through in the digesta. However, this has yet to be demonstrated, and studies of mixed cultures have indicated that protozoa are only inhibited at exceedingly high levels of *Y. schidigera* (Wallace et al., 1994). Assessing direct inhibition is particularly difficult for protozoa because they cannot be grown axenically (Jouany et al., 1988).

It is clear that *Y. schidigera* supplements can indeed influence the growth and metabolism of microbial populations, even in artificial fermentation systems which eliminate any indirect effects that may be exerted via a host animal (Grobner et al., 1982; van Nevel and Demeyer, 1990; Goodall et al., 1979; Peestock, 1979; Valdez et al., 1986; Funk et al., 1988). Our experiments indicate that most of the gut microbiota should be unaffected by *Y. schidigera* supplements but that populations in selected niches may be vulnerable to the accumulation of inhibitory saponins.

Interestingly, in vivo observations have shown that many of the effects of *Y. schidigera* in rats, including those on hindgut variables such as reductions of cecal acetate/propionate and increased urease activity, are caused by the non-butanol-extractable fraction (Killeen et al., 1998). Such effects on the microflora cannot be attributed to either direct steroidal stimulus by 5β-spirostan-3β-ols or direct antimicrobial activity because butanol-washed *Y. schidigera*, which lacks them both, still exerts these effects (Killeen et al., 1998). In summary, it seems that at least these actions of *Y. schidigera* are definitely not mediated by the direct antimicrobial action of saponins **B1**, **B2**, and **B3**.

Other ways in which *Y. schidigera* might exert such effects include surfactant or flocculent effects. The surfactant properties of saponins at very low concentra-

tions are well documented (George, 1965; Price et al., 1987; Hwang and Damodaran, 1994). Furthermore, the fermentation-promoting properties of *Y. schidigera* extracts have previously been associated with flocculation in waste digesters (Peestock, 1979). A surfactant/flocculent action on the feed constituents, thereby altering their digestion rates, would certainly be consistent with the substrate-dependent nature of the effect of *Y. schidigera* on rumen dry matter and nitrogen digestibilities (Goetsch and Owens, 1985). We suggest that this and other possible mechanisms may warrant investigation and that further studies of direct microbe inhibition should focus on those organisms which the properties of *Y. schidigera* saponins indicate may be vulnerable.

ABBREVIATIONS USED

FAB-MS, fast atom bombardment mass spectroscopy; ICD, inhibitory concentration in diffusion; IR, infrared; MIC, minimum inhibitory concentration; MIV, maximum inhibitory volume; NMR, nuclear magnetic resonance; RP-HPLC, reverse phase high-performance liquid chromatography; SEM, standard error of the mean; TLC, thin-layer chromatography.

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