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Sensitivity of sheep intestinal lactic acid bacteria to secondary compounds extracted from *Acacia saligna* leaves

A.Z.M. Salem[a,e,*], P.H. Robinson[b], S. López[c], Y.M. Gohar[d], R. Rojo[a], J.L. Tinoco[a]

[a] *Centro Universitario UAEM-Temascaltepec, Universidad Autónoma del Estado de México, km 67.5 Carretera Toluca-Tejupilco, CP 51300, Estado de México, México*
[b] *Department of Animal Science, University of California, Davis, CA 95616-8521, USA*
[c] *Instituto de Ganadería de Montaña (IGM) CSIC-Universidad de León, Departamento de Producción Animal, Universidad de León, 24071 León, Spain*
[d] *Division of Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt*
[e] *Department of Animal Production, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria, Egypt*

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ABSTRACT

The sensitivity of the lactic acid bacteria (LAB) *Lactobacillus plantarum* and *Enterococcus faecium*), isolated from sheep faeces, to secondary compounds extracted from *Acacia saligna* leaves was investigated. Secondary compounds (SC) extracted from *A. saligna* foliage were: total phenolics (TP), saponins (SP), alkaloids (AK), a methanol extract (ME) and an aqueous extract (AF). All SC were examined with and without PEG for inhibitory effects on *L. plantarum*, *E. faecium* and a LAB mixture (*L. plantarum* + *E. faecium* 1:1). The highest sensitivity (*i.e.*, inhibition zone) occurred in *L. plantarum* to all SC, whereas *E. faecium* was more resistant. All LAB were more sensitive to TP than to other SC, as inhibition zones ranged from 7 to 26 mm^2 for AK and TP in *L. plantarum*, from 5 to 15 mm^2 for the AF and ME in *E. faecium*, and from 5 to 14 mm^2 for AK and TP in the LAB mixture. Adding SC to the bacterial medium reduced ($P<0.05$) growth and lactic acid production. Within each bacterial species, growth, biomass and lactic acid production were lowest ($P<0.05$) when TP extract was added to cultures of *L. plantarum* and the LAB mixture, and when ME was added to cultures of *E. faecium.* Combining both LAB strains increased biomass production with all SC. Resistant colonies increased ($P<0.001$) with TP addition in *L. plantarum*, and with TP, SP and AK in *E. faecium*. PEG decreased inhibitory effects of SC and increased bacterial growth and production of lactic acid and biomass, probably due to its ability to form insoluble complexes with SC. Response to PEG was stronger against TP than other SC, especially *L. plantarum* and the LAB mixture. Overall, *L. plantarum* was more susceptible than *E. faecium* or the LAB mixture to SC, and had a higher response to PEG addition, which reduced toxicity in both bacterial strains. Lactic acid bacteria would be affected to a different extent by SC reaching the small intestine, whereas PEG may neutralize inhibitory effects of SC on these bacterial species.

1. Introduction

Shrub foliages are important sources of forage for ruminants during the dry season when the quality and quantity of green herbages is limited (Salem et al., 2007). Utilization of browse foliages in ruminant feeding is generally limited by

Abbreviations: AA, amino acids; AK, alkaloids; AF, aqueous fraction of lectinspolypeptides and starch; CO, control; LAB, lactic acid bacteria; ME, methanol extract; PEG, polyethylene glycol; SC, secondary compounds; SP, saponins; TP, total phenolics.

* Corresponding author at: Centro Universitario UAEM-Temascaltepec, Universidad Autónoma del Estado de México, km 67.5 Carretera Toluca-Tejupilco, CP 51300, Estado de México, México. Tel.: +1 521 7162695171; fax: +1 521 7162695171.
E-mail address: asalem70@yahoo.com (A.Z.M. Salem).

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secondary compounds (SC; Salem et al., 2006) or non-protein amino acids in *Acacia angustissima* (McSweeney et al., 2005b) due to inhibition of digestive enzymes, formation of complexes with rumen microorganisms and/or direct toxic effects on the animal (Mangan, 1988; Makkar et al., 1995). Nutrient utilization of *A. saligna* leaves is limited due to deleterious effects of SC, such as total phenolics (TP), quinines, flavonoids, terpenoids, essential oils, coumarins, alkaloids (AK), lectins and polypeptides (Cowan, 1999; Salem et al., 2006). Many SC form indigestible linkages with nutrients in the rumen that reverse at the lower pH of the abomasum, thereby releasing the nutrient for post-ruminal digestion. In the lower intestine, SC can disassociate from the nutrient and may react with lactic acid bacteria (Butter et al., 1999). This may be important, as LAB regulate the microbial environment in the small intestine, decrease digestive disturbances, inhibit pathogenic intestinal microorganisms (Brashears et al., 2003), and improve feed conversion and animal health (Windschitl, 1992). These bacteria may also induce anatomical and physiological changes in intestinal cell wall structure, increasing animal resistance to enteropathogenic bacteria (Vanbelle et al., 1999).

The efficiency with which polyethylene glycol (PEG) binds SC, especially TP, has been widely reviewed (Makkar, 2003), and incorporation of PEG into diets high in tannin-rich feedstuffs, such as *Acacia* spp. (Pritchard et al., 1992; McSweeney et al., 2005b), and tree foliages (Salem et al., 2006), has improved ruminant performance.

Our objective was to evaluate the sensitivity of LAB previously isolated from sheep faeces to some SC extracted from *A. saligna* leaves, and ability of PEG to reduce inhibitory effects of SC on LAB activity. This could be important in creating an understanding of how this largely unexamined mechanism of action of PEG neutralizes negative effects of SC to improve the productivity and welfare of ruminants that are fed SC-rich shrubs.

2. Materials and methods

2.1. Isolation and identification of LAB

One gram of fresh faeces, of healthy sheep, was added to 10 ml of sterile De Man et al. (1960) MRS broth media (composition in g/l: 10.0 universal peptone; 5.0 yeast extract; 20.0 D(+) glucose; 2.0 di-potassium hydrogen phosphate; 1.0 polyoxyethylene sorbitan mono-oleate; 2.0 di-ammonium hydrogen citrate; 5.0 sodium acetate; 0.1 magnesium sulfate; 12.0 agar-agar) for cultivation and enumeration of *Lactobacillus* spp. Another gram of fresh faeces was added to 10 ml of M17 broth medium (composition in g/l: 5.0 peptone from soymeal; 2.5 peptone from meat; 2.5 peptone from casein; 2.5 yeast extract; 5.0 meat extract; 5.0 D(+) lactose; 5.0 ascorbic acid; 19.0 sodium β-glycerophosphate; 0.25 magnesium sulfate; 12.75 agar-agar for cultivation and enumeration of *Enteroccocus* spp.; Terzaghi and Sandine, 1975).

Faecal material was streaked onto MRS and M17 agar plates for *Lactobacillus* spp. and *Enteroccocus* spp. selection, respectively. Plates were incubated at 37 °C for 48 h in plastic bags flushed with CO_2 for 30 s. Isolated colonies, 10–15, were selected from each plate and transferred to individual tubes containing 10 ml of MRS or M17 broth medium, which were further incubated at 37 °C for 18–72 h to obtain maximum culture growth. Isolated cultures were re-streaked onto MRS or M17 agar plates for *Lactobacillus* spp. and *Enteroccocus* spp. selection and incubated at 37 °C for 48 h until isolated colonies of the same morphology were obtained. Pure colonies were Gram stained for preliminary identification. Isolated cultures were maintained as stocks in MRS broth supplemented with 100 ml/l sterile glycerol. Isolates were sub-cultured in MRS or M17 broth medium at 37 °C for 24–48 h (culture activation) before use in further studies.

Lactobacillus plantarum and *Enterococcus faecium* were identified by morphological and physiological tests (Garvie, 1986; Kandler and Weiss, 1986). Gram-positive cocci (*E. faecium*) were tested for growth in M17 broth medium. Gram-positive rod shaped isolates (*L. plantarum*) were examined for growth and CO_2 production in MRS broth.

2.2. Treatments

Previously isolated LAB (*i.e.*, *L. plantarum*, *E. faecium*) from sheep, and their 1:1 mixture, were tested with each of the secondary compounds (*i.e.*, SC) extracted from *A. saligna* leaves in g/kg DM of leaves (*i.e.*, methanol extract (ME), total phenolics (TP, 61.0 g), saponins (SP, 23.5 g), alkaloids (AK, 3.2 g), and the aqueous fraction (AF, 68.0 g; *i.e.*, lectins, polypeptides and starch; see review of Cowan, 1999) in the absence or presence of PEG (MW 4000). The PEG stock solution (20 mg/ml) and each SC stock solution (20 mg/ml) were mixed 1 ml with 1 ml just before use. Stock solutions of each of the SC were prepared by dissolving them in dimethylsulfoxide (DMSO), except TP extracts, which were dissolved in aseptic propylene glycol. The bacterial strains (*i.e.*, *L. plantarum*, *E. faecium*, and the mixture) were activated by incubation at 37 °C for 24 h and turbidity was adjusted to optical density 600 (OD_{600}) = 1 with sterilized broth medium before use.

2.3. Extraction of SC

Leaves of *A. saligna* were randomly and manually harvested from different parts of both young and mature leaves during the dry season and prepared for SC assays according to Salem et al. (2006). For ME, plant materials were dried at 40 °C for 72 h, homogenized using a blender and ground to pass a 1 mm screen. Powdered plant tissue (100 g) was extracted in 800 ml/l methanol and evaporated under reduced pressure at 40 °C. The extract was dried overnight at room temperature (25–30 °C) and stored in the dark at 4 °C until use. Extraction processing used funnel separation. For the TP component, 1 g of the ME was fractionated with ethyl acetate, while the AK extract was extracted using the dried samples extracted with ethanol and