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Determination of in vitro isoflavone degradation in rumen fluid

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ABSTRACT

The aim of this study was to determine the degradation of dietary isoflavones in rumen fluid under 2 feeding regimens. The experiments were performed in vitro using a rumen fluid buffer system. The rumen fluid was taken from cows fed either a hay diet or a concentrate-rich diet (the diet consisted of 34.6% maize silage, 17.6% haylage, 12.8% alfalfa hay, and 35.0% supplemental mixture on a dry matter basis). As a source of isoflavones, 40% soybean extract (Biomedica, Prague, Czech Republic) at levels of 5, 25, 50, and 75 mg per 40 mL of rumen fluid was used. Samples of soybean extract were incubated in triplicate at 39°C for 0, 3.0, 6.0, 12.0, and 24.0 h in incubation solution. The metabolism of daidzein and genistein was faster under concentrate-rich diet conditions. In general, production of equol started after 3 to 6 h of incubation and reached the highest rate after approximately 12 h of incubation regardless of the type of diet or concentration of extract. In most of the experiments, production of equol continued after 24 h of incubation. Generally, equol production was greater under the hay diet conditions. Furthermore, experiments with higher amounts of added soybean extract revealed possible inhibitory effects of high levels of isoflavones on the rumen microflora.
Key words: isoflavones, equol, rumen, cattle diet

INTRODUCTION

Ruminant feedstuffs may contain isoflavones, which are known to have weak estrogenic activity (Zhengkang et al., 2006). Two major sources of isoflavones are commonly used in the nutrition of dairy cows: soy (*Glycine max*) and red clover (*Trifolium pratense* L.). Soybeans and soybean products—common components of dairy cattle diets (Chouinard et al., 1997)—are rich in the isoflavones genistein and daidzein (Chan et al., 2009), but these can be further metabolized by microorganisms in the gastrointestinal tract to more potent metabolites (Schoefer et al., 2002; Wang et al., 2005; Wocławek-Potocka et al., 2013). In the rumen, daidzein is further metabolized to equol and genistein is metabolized to *p*-ethyl-phenol (Lundh et al., 1990; Lundh, 1995). Although *p*-ethyl-phenol is an inactive metabolite (Setchell et al., 2002), equol is more bioactive than its precursor daidzein, with higher estrogenicity (Kostelac et al., 2003), antioxidant activity (Turner at al., 2004), and antiandrogenic properties (Lund et al., 2004).

The rumen is a phylogenetically complex ecosystem, where microorganisms convert feedstuffs into microbial biomass and fermentation end products that can be utilized by the host animal (Kong et al., 2010). Three taxonomic groups of microorganisms—bacteria, protozoa, and fungi—carry out this digestion process in the rumen. Edwards et al. (2004) suggested that the rumen might contain 300 to 400 bacterial species. This microbiota is highly responsive to many factors, especially to changes in the feeding regimen and in the composition of the diet, to the health of the host animal, or to antibiotic use (e.g., Hungate, 1966; Stewart and Bryant, 1988; Tajima et al., 2000, 2001).

The adaptation of the rumen to a high-grain diet principally involves increases in the populations of bacteria capable of utilizing lactic acid (Counotte and Prins, 1981). Using quantitative PCR, Tajima et al. (2000, 2001) detected gram-positive bacteria belonging to the *Selenomonas-Succiniclasticum-Megasphaera* group with *Selenomonas ruminantium* and *Megasphaera multiacida* being the most numerous group in the grain diet microbiota. Furthermore, they found that numbers of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in animals on a hay diet were up to 20 times higher than those of animals for which the diet was switched from hay to grain, and concluded that the dynamics of the truly fibrolytic rumen bacteria correlated strongly with

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the diet change (Tajima et al., 2001). Such changes in the profile of rumen microbiota may affect the metabolism of certain substances in the rumen.

Recent studies suggest that isoflavones may have beneficial (Zhu et al., 2002; Mao et al., 2007) or inhibitory (Verdrengh et al., 2004; Mukne et al., 2011) effects on rumen microorganisms. Conversely, the effect of diet on the metabolism of isoflavones has been documented in some studies. Gagnon et al. (2009) and Petit et al. (2009) observed that PUFA affected bacteria with β-glucuronidase activity such as *Ruminococcus*, *Bacteroides*, *Bifidobacterium*, and *Eubacterium*, which may be involved in deconjugation of isoflavone glycosides. Furthermore, the level of conversion of daidzein to equol can be influenced by feed intake (Njåstad et al., 2014) or by intake of some nutrients such as nonstarch polysaccharides (Ohta et al., 2002; Lipovac et al., 2015), fat (Rowland et al., 2000), fiber, or a higher proportion of plant proteins (Lampe et al., 1998; Nielsen and Williamson, 2007).

Thus, the aim of the study was to determine the effect of type of diet and concentration of dietary isoflavones on their metabolism in the rumen using in vitro methods.

MATERIALS AND METHODS

Experimental Design, Animals, and Diets

The experiments were performed in vitro using a rumen fluid buffer system. As a natural source of isoflavones, 40% soybean extract (Biomedica, Prague, Czech Republic) in powder form was used, which contained daidzein, genistein, and glycitein in the ratio 1:0.070:0.003. In the whole experiment, 4 amounts of soybean extract, 5, 25, 50, and 75 mg per 40 mL of the buffered rumen fluid, were used to investigate the kinetics of isoflavone degradation. As a source of rumen fluid, nonlactating cows fed a hay diet or lactating dairy cows fed a concentrate-rich diet were used. The concentrate-rich diet consisted of 34.6% maize silage, 17.6% haylage, 12.8% alfalfa hay, and 35.0% supplemental mixture (on a DM basis). The supplemental diet contained (g/kg): barley 266.0; oat 266.0; sugarbeet chippings 150.0; extruded rapeseed meal 282.2; rapeseed oil 10.5; sodium chloride 5.5; dicalcium phosphate 7.5; limestone 10.5; sodium bicarbonate 1.0; blend-s minerals 0.5 (Biokron s.r.o., Blučina, Czech Republic); and blend-s vitamins 0.5 (Biokron s.r.o.). Animal handling was performed according to current Czech legislation (Act No. 246/1992 Coll. to protect animals against cruelty, as amended). Rumen fluid was collected 3 h after morning feeding via rumen cannula (2 dry rumen-cannulated cows) or via a flexible, stainless-steel stomach tube (2 intact lactating cows), placed in bottles with CO_2, and transferred to the laboratory in a heat-stable box. Immediately, pH was measured and the rumen fluid from the 2 cows on the same diet was mixed and filtered through 4 layers of cheesecloth. The fluid was then mixed with a prewarmed buffer (39°C) in a ratio of 1:1. The buffer (pH 6.9) was composed of solutions A and B (A:B 5:1) with following compositions: solution A = 10.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 0.1 g/L $CaCl_2 \cdot 2H_2O$, and 0.5 g/L urea; solution B = 15.0 g/L Na_2CO_3 and 1.0 g/L $Na_2S \cdot 9H_2O$.

The incubation process was conducted in 90-mL (thick-walled) glass tubes containing 40 mL of incubation solution, 0.5 g of the respective diet (hay or concentrate-rich) ground through a 1-mm screen, and soybean extract at 4 levels as noted above, with each level in triplicate. Tubes were purged with CO_2 before being sealed with rubber stoppers with a Bunsen gas release valve. Then, samples were incubated at 39°C for 0, 3.0, 6.0, 12.0, and 24.0 h with gentle manual shaking 3 times per day. For each type of diet, in vitro incubations were conducted one at a time.

Sample Preparation

The samples were taken after 0, 3.0, 6.0, 12.0, and 24.0 h of incubation from separate tubes so that only one sampling was done from each tube. Collected samples were cooled in ice water, centrifuged to separate off the microbial mass (5,000 × *g*, 15 min, 5°C), and kept frozen at −20°C until analyses. Each run was done 3 times. Fermented samples were analyzed for the content of isoflavones (daidzein, glycitein, genistein) and the metabolite equol.

Sample preparation was done according to the method described in our previous paper (Kasparovska et al., 2016a) with the following modifications. The sample of rumen fluid was extracted twice to ethyl acetate and evaporated to dryness. The residue was thoroughly dissolved in 1 mL of water and the acids were removed from the sample by solid-phase extraction (Chromabond SB, Macherey Nagel, Düren, Germany). The samples were evaporated to dryness, dissolved in 1 mL of 50% methanol, and filtered through a 0.20-μm filter.

Analytical Procedure

The soybean extract added to the reactions contained primarily daidzein, genistein, and glycitein. First, we analyzed a part of the samples using liquid