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Short- and long-term effects of low estrogenic subterranean clover on ewe reproductive performance

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ABSTRACT

This study examined the short- and long-term effects on the reproductive performance of sheep by subjecting them to varieties of subterranean clover (*Trifolium subterraneum*), containing a low content of total phytoestrogens and formononetin. In the first trial, 30 ewe lambs of the Sarda breed (initial mean live weight 16.4 ± 1.3 kg) were divided into three groups of 10 animals each and maintained separately on pastures for 60 d, with alfalfa (*Medicago sativa* L.), oats (*Avena sativa*), and subterranean clover with a phytoestrogen content ranging from 10.21 mg/g on a dry matter basis (DM) at the onset, to 0.90 mg/g DM at the end of the trial, respectively. All three pastures were integrated to make them isoproteic and isoenergetic. At the end of the grazing period, the animals were pooled into one group and fed the same phytoestrogen-free diet. No differences were recorded between the groups with regard to the development of the reproductive system as monitored by transvaginal ultrasonography, fertility, fecundity, breeding performance of the ewes, or with regard to the birth weight and conformation of the lambs. Puberty was attained earlier by the animals fed on subterranean clover, while the lambs also weighed more. In the second trial, 24 ewe lambs of the Comisana breed (initial mean live weight 25.0 ± 3.4 kg) were divided into two groups of 12 animals each. All animals were fed ad libitum for a 20 months period with fresh-cut or dried subterranean clover (phytoestrogen content of 0.88 and 0.81 mg/g DM, respectively); or a control non-estrogenic diet, based on fresh-cut or dried Italian ryegrass (*Lolium multiflorum* Lam.). The two diets were integrated so to be isoenergetic and isoproteic. No differences were recorded between the groups with regard to the attainment of puberty, although the animals fed clover were heavier at puberty. The development of the reproductive system, fertility, fecundity and breeding performance in both lambings, the interval between lambings, and body weight and conformation of the lambs did not show differences. In both trials, neither non-degraded phytoestrogens nor equol (metabolite of formononetin) were recorded in the blood of the ewes. The only metabolite present was *p*-ethylphenol. All animals fed subterranean clover-based diets, showed a significantly higher ADG, compared to those fed the control diets. The results of this study indicate that the levels of total phytoestrogens and formononetin in the new varieties of subterranean clover do not negatively affect the reproduction of sheep and appear to improve the growth rate of the animals.

1. Introduction

Researchers have noted disturbances in ruminant reproduction, emanating from the ingestion of subterranean clover (SC), containing high quantities of phytoestrogens (PEs) and isoflavones that have an estro-

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genic activity (Adams, 1977, 1990, 1995; Firth et al., 1977; Kallela et al., 1984).

Formononetin and its ruminal metabolite, equol, cause the most negative effects of all the PEs, with equol having the greater estrogenic activity (Shutt and Braden, 1968; Shutt et al., 1970; Lloyd Davies and Hill, 1989). Rumen micro-organisms extensively transform the other estrogenic isoflavones present in the SC, principally genistein and biochanin-A, into a *p*-ethylphenol – a substance with no hormonal activity (Lundh et al., 1990). However, recent in vitro studies have shown that *p*-ethylphenol can stimulate the production of prostaglandin-F2α in the corpus luteum in a way similar to estrogen (Woclawek-Potocka et al., 2006).

Due to the close structural similarity of the PEs (phytoestrogens) to mammalian steroid hormones, some researchers have speculated that in animals, PEs could have anabolic effects, similar to those of the sex hormones (Trenkle and Borroughs, 1978; Greiner et al., 2001; Moorby et al., 2004). Furthermore, the chemical structure of the isoflavones and their metabolites confer the capacity on these substances to bind themselves to estrogen α and β receptors (Kuiper et al., 1998) where they display both estrogenic and anti-estrogenic activity (Cassidy and Faughnan, 2000). Recent studies in different animal species have highlighted the numerous biological effects of PEs, such as, e.g., higher plasma growth hormone (GH) and insulin-like growth factor (IGF-1) concentrations (Moorby et al., 2004); the stimulation of GH production in the central nervous system (CNS) (Misztal et al., 2007); increased blood calcium levels and bone density, and an improved feed conversion index (Ni et al., 2007). The in vivo effects of PEs have been the subject of a series of tests in laboratory animals (mice) and monogastrics (rabbits), in which, in addition to their normal diet, these animals were fed a lyophilized alcoholic extract of SC (subterranean clover). In all the tests, the animals that received PEs demonstrated an average daily gain significantly higher than the controls (Pace et al., 1994; Pace and Settineri, 1996).

Studies involving Friesian calves (both bull and castrates) and fed a diet based on fresh SC (total PEs 0.92% on a DM basis), have highlighted the positive effect on the growth in bull calves. The castrated animals exhibited a positive effect regarding ADG only in the first month, before showing an abrupt decline during the subsequent month (Settineri et al., 2002). Furthermore, prepubertal Friesian heifers pasture fed SC (total PE of 0.17% on a DM basis), exhibited an ADG that was significantly higher than that of the control group – with no effect on the onset of puberty (Pace et al., 2001). Currently, new varieties of subterranean clover (SC) have been selected for a reduced content not only in formononetin, but also in phytoestrogens (PEs), with values that do not exceed 0.5% to 1% DM. The aim of the present study was to verify whether these levels of PEs, and in particular of formononetin (administered over varying periods of time), are associated with a reduction in the fertility of ewes, alterations in the reproductive organs, have negative effects on the course of gestation and lambing, or cause morphologic modifications to the offspring.

2. Materials and methods

2.1. Reagents and materials

Standards of the isoflavones genistein (4′,5,7-trihydroxyisoflavone), formononetin (7-hydroxy-4′-methoxyisoflavone), daidzein (4′,7-dihydroxyisoflavone), and biochanin-A (5,7-dihydroxy-4′-methoxyisoflavone) were obtained from Sigma–Aldrich (Milan, Italy). *P*-ethylphenol, equol (4′,7-isoflavandiol), methanol, and water, the latter both being HPLC grade, while the other organic solvents of analytical grade, also supplied by Sigma–Aldrich (Milan, Italy). Standard stock solutions of each compound were prepared by dissolving the standards in a suitable quantity of methanol. Working solutions containing each of the 4 compounds were prepared by dilution of the standard stock solutions, and stored at −20 °C. Glucuronidase–sulfatase of *Helix pomatia* was purchased from Sigma–Aldrich (Milan, Italy). The cartridge C18 employed for solid phase extraction of the plasma was obtained from Waters (Milford, MA, USA) and the 0.45 μm PTFE filters acquired from Millipore (Billerica, MA, USA). Plasma progesterone (P4) assays were performed using a DSL-3900 P4 RIA Kit (DSL, Webster, TX, USA), and values were recorded in a gamma-counter (Cobra II, Packard A, Camberra Company (USA).

2.2. Animals and experimental design

2.2.1. Trial 1

In trial 1, 30 ewe lambs of the Sarda breed, with an initial mean live weight of 16.4 ± 1.3 kg, were subdivided into three groups and fed isoproteic and isoenergetic diets for 2 months, as follows: Group A, grazing on alfalfa + 300 g/d maize grain + 200 g/d sunflower meal; Group B, grazing oats + 250 g/d maize grain + 250 g/d sunflower meal; and Group C, grazing subterranean clover (SC) + 300 g/d maize grain + 200 g/d sunflower meal. Three cultivars of SC (Karridale, Northam and Nuba, purchased from S.A.G., Olbia, Italy), selected on the basis of their low PEs and formononetin content, were sown in autumn. When the plants reached a height of 15 cm, samples of the whole plant and of the leaves were collected every 2 weeks. At the end of the first grazing period, the lambs were reunited into one group, and two fertile rams introduced. The group was then fed the same non-estrogenic standard diet for 8 months. During the grazing trial and the three following weeks, blood was sampled weekly by jugular puncture at three specific times on the same day (08h00, 13h00, and 18h00). The plasma samples were then used to determine the concentrations of PEs and metabolites and progesterone (P4) concentration. Ewe lambs were considered to have attained puberty when the P4 plasma levels were higher than 1.0 ng/ml.

A month after the introduction of the rams, tests were initiated to confirm pregnancies and the status of the reproductive system by means of transvaginal ultrasonography. The lambing date was also used to verify the breeding times. Gestation length was assumed to be 150 d.

The course of the lambing and the litter number, birth weight, and conformation of the lambs born were recorded.

2.2.2. Trial 2

In trial 2, 24 ewe lambs of the Comisana breed with an initial mean live weight of 25.0 ± 3.4 kg were subdivided into two groups and fed for a 20 month period in a sheepfold on isoproteic and isoenergetic diets, as follows: Group A: SC (cultivars Antas, Limbara and Trikkala, purchased from S.A.G., Olbia, Italy) ad libitum + 300 g/d maize grain + 250 g/d sunflower meal; and Group B: (control, PE-free); Italian ryegrass (IR) ad libitum + 250 g/d maize grain + 350 g/d sunflower meal. During the period April–June (spring), the forage was cut daily and provided fresh. Subsequently, the same forage was fed as hay. During the trial, weekly blood samples were taken from the jugular vein for the determination of the plasma PE and their metabolite concentrations and, up until the attainment of puberty, for the level of P4. The ewes were considered to have attained puberty when plasma P4 levels were higher than 1.0 ng/ml.

Two fertile rams were then introduced into each of the two groups, and after a month, transvaginal ultrasonography was used on a monthly basis, to confirm pregnancies and evaluate the condition of the reproductive system. The lambing date was used to verify the breeding times while the gestation length was assumed to be 150 d. Data were recorded regarding the course of the births, the number of offspring, birth weight and conformation of the lambs born. For verification of the long-term effects of PEs on fertility and reproduction, the ewes were mated again