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Phyto-estrogenic activity of protein-rich feeds for pigs

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SUMMARY

In this report the levels of phytoestrogens in new protein-rich feed crops for pigs, like pea (*Pisum sativum*), lupin (*Lupinus alba*, *Lupinus luteus* and *Lupinus angustifolius*), broad bean (*Vicia faba*) and quinoa (*Chenopodium quinoa*) and their possible effects on the reproduction performance of sows are described.

Phytoestrogens are plant-derived compounds that can exert an estrogenic or anti-estrogenic action and thus influence fertility and reproduction. Main phytoestrogens are isoflavones (genistein and daidzein), coumestans (coumestrol) and lignans (matairesinol [MAT] and secoisolariciresinol [SECO]).

Peas contain moderate levels of isoflavones and some SECO, almost no MAT and coumestrol. Broad beans contain low to moderate levels of isoflavones, moderate levels of MAT and no coumestrol. Lupin seeds contain only trace amounts of genistein. The highest amount of flavonoid glycosides, expressed as genistein-7-O glucoside equivalents, was found in the root and leaves of lupin, reaching 650 and 600 mcg/g fresh weight. Seeds of the white lupin normally contain only trace amounts of isoflavonoids, but when soaked with water larger amounts of genistein are synthesized.

Quinoa is food crop, native to the Andean regions of South America. Although its use as feed and food is extensively described, isoflavonoids are seldom mentioned. Flavonoids were found in five species of *Chenopodium*, consisting of quercetin, kaempferol and isorhamnetins. Flavonoid glycosides such as kaempferol glycosides, mauritianina and minor components have been identified, but no isoflavones were mentioned in literature.

Although phytoestrogens are known for their effects on fertility and reproduction in laboratory and production animals, for pigs there are very few data. This is in contrast to the amount of data on estrogenic effects of feeds contaminated with the mycotoxin zearalenone, for which pigs appear very susceptible.

As compared to soybeans, the levels of phytoestrogens in peas, broad beans, lupins and quinoa are very low. Since soybean meal is included in conventional pig feed up to 25 % without reported effects on fertility, it is not likely that the much lower levels of phytoestrogens from peas, broad beans and quinoa will be able to influence fertility negatively.

1 INTRODUCTION

In the EU project “Quality Low Input Food”, WP 4.4.2 concerns “Suitability of different organically grown protein crops for optimising pig rations”. In WP 4.4.2.1. a literature review and screening of different candidate protein crops is performed. In this report the levels of phytoestrogens in protein crops and their possible effects on reproduction of sows are described.

Organic farming is stimulated to use organically produced feed for their animals. The use of conventionally produced protein sources is not allowed in organic pig production, because they are not organically grown. Legume crops are a choice crop as a protein source for organic animal feed, because of the high protein levels in the seeds. However, most legume crops contain antinutritional factors (ANFs).

Some new legume crops contain reduced levels of some of the ANFs (e.g. white lupine). Also other crops such as quinoa could be useful as protein source for organic animal feed.

As possible protein sources for organic pig feed the following crops are investigated: pea (*Pisum sativum*), lupin (*Lupinus alba*, *Lupinus luteus* and *Lupinus angustifolius*), broad bean (*Vicia faba*) and quinoa (*Chenopodium quinoa*). These crops have high protein levels and a favourable amino acid composition, since they can be easily supplemented with grains.

All leguminoses contain ANFs, substances that reduce the digestibility and utilisation of the feed. Main ANFs are: alkaloids, tannins, phytate, protease inhibitors, lectins, saponins, oligosaccharides and phytoestrogens.

Phytoestrogens are estrogenic compounds found in plants. According to the classical definition phytoestrogens are compounds that exert estrogenic effects on the central nervous system, induce oestrus and stimulate growth of the genital tract of female animals (Kurzer and Xu, 1997). Most phytoestrogens don't have a steroid structure, but are phenols with a chemical structure that resembles estrogens. Plant estrogens may, when present in high levels, affect the fertility of production animals. In Australia and New Zealand sheep grazing on estrogenic clover pasture have shown symptoms of “clover disease”, a fertility disorder due to changes in the genital tract (Adams, 1995), induced by estrogenic compounds in subterranean and red clover.

The estrogenic effect usually derives from isoflavones, coumestans and lignans.

Another source of estrogenic activity is the estrogenic mycotoxin zearalenone from the fungus *Fusarium roseum* (Diekman et al., 1992). In this report estrogenic mycotoxins are not discussed since their effects are described in an earlier report (Groot, 2003).

In this report the presence of plant estrogens in pea, lupine, broad bean and quinoa and their possible phytohormonal effects on the animals are investigated.

2 BACKGROUND

2.1 General

Phytoestrogens can be divided into isoflavones, lignans and coumestans (Dixon, 2004). Other feed estrogens are the resorcylic acid lactones such as zearalenone, which are secondary mould metabolites mainly from *Fusarium* species. Phytoestrogens have a weak estrogenic potency, ranging from 1/500 to 1/10000 to that of 17Beta-estradiol.

There are over 300 plants identified that have sufficient estrogenic activity to initiate oestrus in animals (Farnsworth et al., 1975). Isoflavones and coumestans are the most common estrogenic compounds in this list.

Levels of phytoestrogens in plants vary and depend on the species variety, the time of year, growth conditions, light, climate, plant part and the occurrence of infections (Lindner, 1978). Moreover, there are differences between fresh and dried material, influences from storage conditions (Kallela, 1975) and most important, the presence of mould infections can add phytoestrogenic activity from mycotoxins such as zearalenone.

2.2 Isoflavones

Isoflavones are secondary metabolites produced mainly in leguminous plants. They play a role in both positive and negative plant-microbial interactions. On one hand they function in the symbiotic relationship between plant and the rhizobial bacteria for the formation of nitrogen fixing root nodules. On the other hand they function in the disease resistance response, where they are synthesized as defence compounds against microorganisms and insects.

Main estrogenic isoflavones are genistein, daidzein, biochanin A and formononetin.

Isoflavones undergo extensive metabolism in the intestinal tract (Livingston, 1978). Biochanin A is the precursor of genistein, formononetin is the precursor of daidzein, and daidzein can be metabolised into equol. Isoflavones can be found in four chemical forms, the unconjugated forms or aglycones, the glucoside form, acetylglucosides and malonylglucosides. Isoflavones have functional similarity to natural estrogens and can weakly bind to oestrogen receptors, causing competition with natural estrogens (Kelly et al., 1993).

There are few data concerning metabolism of isoflavones in pigs (Lundh 1995). It is suggested that as with other monogastric animals, glycosides are cleaved by intestinal bacteria into sugars and the biologically active isoflavones. After absorption, isoflavones are transported to the liver where they are removed from the portal blood. However, a percentage of the isoflavones escapes uptake by the liver and enter the peripheral circulation. The isoflavones are excreted primarily via the kidneys. In a feeding experiment with pigs fed a mixture containing 20 % red clover, formononetin was absorbed very rapidly and reached a total (conjugated and free) maximum level in the blood within one hour. This suggests that absorption already takes place in the stomach (Lundh, 1995). Daidzein and equol showed similar patterns, but lower levels than formononetin. About 55 % of the ingested formononetin was excreted with the urine within eight hours post feeding.

2.3 Coumestrol

Coumestrol is the most potent of the coumestans and has a higher binding affinity for the estrogen receptor than genistein (Shemesh et al., 1972). Coumestrol can be found in low concentrations in legumes such as lucerne and alfalfa and in clover. However the concentration of coumestrol in both clover and alfalfa can increase as a response to fungal disease, as part of the plant defence mechanism against foliar pathogens (Bickoff 1968). Otherwise coumestrol is usually not present in sufficient concentrations in pasture crops to affect grazing animals (Shutt 1976).

2.4 Lignans

Lignans are minor constituents of many plants, as they are the building blocks of cell walls. Main lignans are matairesinol (MAT, precursor of enterolactone) and secoisolariciresinol (SECO, precursor of enterodiol), lariciresinol and isolarisoretinol. The mammalian lignans enterolactone and enterodiol are the products of colonic bacterial metabolism of the plant lignans MAT and SECO. The lignans are mainly present as glycosides in the plant and are hydrolysed in the proximal colon (Mazur and Adlercreutz, 1998).

2.5 Mode of action of phytoestrogens

Phytoestrogens can exert their effects on reproductive function in many ways (Rosselini et al., 2000). First way is by binding to the oestrogen receptor (ER) and thus competing with the natural oestradiol. The binding affinity to the receptor can vary; genistein has a very high binding affinity whereas biochanin A has not. Phytoestrogens bind to both ER α and ER β . Genistein has also shown to inhibit tyrosine kinase activity and to down-regulate EGF-receptor autophosphorylation. It is suggested that the oestrogen-like growth effects of genistein are largely mediated through its inhibitory effects on these signal transduction pathways. Because of its high receptor binding affinity, genistein may also block the effects of oestradiol and so induce anti-estrogenic effects. Another way of inhibiting oestradiol effects is by inhibition of the enzyme (hydroxysteroid oxidoreductase), which metabolises oestrone to oestradiol, thereby reducing the synthesis of oestradiol.

Coumestrol binds with high affinity to both ER α and ER β and acts as an atypical oestrogen that does not stimulate uterine cellular hyperplasia. On the other hand it has been shown that coumestrol induced a 3-fold induction of ER and thereby increased the sensitivity of the tissues for endogenous oestradiol. Phytoestrogens are shown to increase plasma concentrations of steroid binding protein and decrease the plasma levels of free oestrogen. They may also inhibit the binding of oestradiol and testosterone to steroid binding protein, which results in increased clearance and metabolism of these steroids. It is also suggested that phytoestrogens may influence CYP450 enzyme activities that regulate oestradiol metabolism. Isoflavones also bind to the AhReceptor. Finally, the action of phytoestrogens may depend on the levels, showing estrogenic activity at low levels and anti-estrogenic activity at low levels. Phytoestrogens are capable of inducing both estrogenic and anti-estrogenic activity, which are in part mediated by ER and AhR, but also indirect and independent of receptor binding (Rosselini et al., 2000).

3 LEVELS OF PHYTOESTROGENS

In table1 data on levels of different phytoestrogens in: pea (*Pisum sativum*), lupine (*Lupinus alba*, *Lupinus luteus* and *Lupinus angustifolius*), broad bean (*Vicia faba*) and quinoa (*Chenopodium quinoa*) are presented based on review of literature.

Most data on phytoestrogen levels concern soybeans or soy products; Table 1 summarizes the data found for the crops we investigated and soybean.

Table 1. Phyto-oestrogen content of feed crops

Botanical name	Isoflavones		Lignans		Coumestans
	Daidzein	Genistein	Seco-isolariciresinol (SECO)	Matairesinol (MAT)	Coumestrol
<i>Pisum sativum</i>	4-11 x 10 ⁻² (¹) 41-130 x10 ⁻³ sd 9(⁴) 3.7-11.3 x10 ⁻² (⁵)	0-23 x10 ⁻² (¹) 144 -347 x10 ⁻³ sd 16(⁴) 0.0-22.8 x10 ⁻² (⁵)	3-13 x10 ⁻² (¹) 2.8-12.8 x10 ⁻² (⁵)	0-traces ¹ 0.0-traces ⁵	0.4-06 ² 0.0-traces ⁵
<i>Lupinus albus</i>		5.60 ³			
<i>Lupinus luteus</i>		+			
<i>Lupinus angustifolius</i>	-	+			
<i>Lupinus mutabilis</i>	Traces ⁵	2.42 x 10 ⁻² (⁵)	3.1 x 10 ⁻² (⁵)	0.0 ⁵	0.0 ⁵
<i>Vicia faba</i>	74 x 10 ⁻³ sd 8(⁴) 15.8-31.8 x 10 ⁻² (⁵)	59 x 10 ⁻³ sd 12(⁴) traces ⁵	26-31.8 x10 ⁻² (⁵)	traces-131.0 x 10 ⁻² (⁵)	0.0 ⁵
<i>Chenopodium quinoa</i>					
<i>Glycine Max</i> (soy bean)	10-41.200(⁵)	26-46.300(⁵)	13.3-273(⁵)	traces	traces-185(⁵)

1 In mcg/g dry weight, (Mazur and Adlercreutz, 1998)

2 In mcg/g (Livingstone, 1978)

3 In mcg/g dry seeds (Sirtori et al., 2004)

4 In mcg/g dry weight (Liggins et al., 2000)

5 In mcg/g (Mazur et al., 1998)

Soybeans (seed) have the highest isoflavone contents and contain approximately 200-2600 mcg/g dry weight daidzein and genistein (Mazur and Adlercreutz, 1998). Also SECO is relatively high, whereas MAT is low and coumestrol varies from traces to high levels.

Pisum sativum

As compared to other feed crops peas (seed) contain moderate levels of isoflavones and some SECO, but almost no MAT and coumestrol.

Vicia faba

Broad beans (seed) contain low to moderate levels of isoflavones, moderate levels of MAT and no coumestrol.

Lupines

Lupine seeds only contain trace amounts of genistein (Katagiri et al., 2000; Sirtori et al., 2001 and 2003). In the leguminous plants, isoflavonoid glycosides are partially present as acylated conjugates. Glycosides acylated with aliphatic acids are labile compounds that can easily deacylate (Bednarek et al., 2004). White lupine (*Lupinus albus*) seeds have a very low content of isoflavones (Sirtori et al., 2001 and 2003).

Lupinus albus contains 4 isoflavonoid aglycones, 14 isoflavonoid glycosides, 4 flavonol glycosides and flavone glycoside as measured with LC/ESI/MS (Bednarek et al., 2001).

Among them are 2 hydroxy genistein 7,4,-O- diglucoside, genistein 7,4,-O- diglucoside, genistein 7-O-glucoside-4 O-(6-O Malonyl) glucoside, 2 hydroxygenistein, genistein 7-O glucoside, 2 hydroxygenistein 7-O-(6-O Malonyl) glucoside, genistein 4-O glucoside, genistein 4-O-(6-O-malonyl) glucoside, genistein 7-O-(6-O-malonyl) glucoside, 2 hydroxygenistein and genistein. The highest amount of flavonoid glycosides expressed as genistein 7-O glucoside equivalents were found in the root and leaves of lupines, reaching 650 and 600 mcg/g fresh weight. Sakasai identified two novel 2-hydroxygenistein dimers in the root of white lupin (Sakasai et al., 2000).

There are different publications on flavonoid profiles as response to biotic and abiotic stimuli (Bednarek et al., 2003) but levels of isoflavones in seed are difficult to find. The structure of flavonoids in the aerial parts of yellow lupines has been described (Franski et al., 1999). The only isoflavonoid of interest that was found was genistein.

Seed of the white lupine normally contains only trace amounts of isoflavonoids, but when soaked with water large amounts (from 0.07 to 5.19 $\mu\text{mol/gr. fr. wt.}$) of genistein are synthesized (Katagiri et al., 2000).

In the pods of white lupines the amount of 20hydroxygenistein and genistein increased from trace to 3,4 mcmol/g fresh weight during ripening, whereas genistein glycosides increased from trace to 0.5 mcmol/g fresh weight (Katagiri et al., 2000). Total isoflavones increased from 0.5-to 10.3 mcmol/g fresh weight, but in the seeds only traces of genistein were detected.

In yellow lupines, two glycosyl flavonoids were detected (Katagiri et al., 2002) genistein 8-C-beta-glucoside and apigenin 7- O-beta-neohesperidoside together with genistein 7-0-beta-glucoside.

In *Lupinus mutabilis* two new isoflavones were detected in the flour (Dini et al., 1998), which were named mutabilin (3-methoxy-5 hydroxy-7-0-beta-D glucosylisoflavone) and mutabilein (3-methoxy-5,7-dihydroxyisoflavone).

Chenopodium quinoa

Quinoa is a lesser-known grain, native to the Andean regions of South America. Although its use as feed and food is extensively described (Ahamed et al., 1997), isoflavonoids are seldom mentioned. Flavonoids were found in five species of *Chenopodium*, consisting of quercetin, kaempferol and isorhamnelins (Bahrman et al., 1985). Flavonoid glycosides such as kaempferol glycosides, mauritianina and minor components have been identified (De Simone et al., 1990), but no isoflavones were mentioned in literature.

4 DISCUSSION

The levels of phytoestrogens in the investigated feed crops pea (*Pisum sativum*), lupines (*Lupinus alba*, *Lupinus luteus* and *Lupinus angustifolius*), broad bean (*Vicia faba*) and quinoa (*Chenopodium quinoa*) are low as compared to soybean, which is widely used in conventional pig feed.

There is scarce literature on effects of phytoestrogens in pigs. In vitro genistein exerts stimulatory effects on reproductive processes (Makarevich et al. 1997), but no extrapolation to in vivo has been made so far. Effects of extra dietary isoflavones on growth and carcass traits were investigated (Payne et al., 2001) but no effects on reproduction were mentioned.

Most data on phytoestrogenic effects in pigs concern intoxications with the mycotoxin zearalenone (Diekman et al., 1992; Dacasto et al., 1995; Riley, 1998).

In a review on effects of nutrition on male fertility, Brown (1994) states that for male animals it is almost impossible to ingest enough estrogenic plants to impair fertility. For female animals this is different (Adams, 1995), because dietary estrogens can affect the morphology of the genital tract, oestrus cycling, embryo development and reproductive function (McEvoy et al., 2001).

High levels of soybeans in the diet of female Wistar rats led to growth depression, earlier vaginal opening, increase of the weight of the uterus and histological changes in the ovaries and genital epithelia (Gallo et al., 1999). In this study, rats were fed with a standard cereal based diet or with that diet supplemented with 0.7, 1.2 or 2.4 % soy extract from weaning until 7 day postpartum. Most obvious changes were observed in the high dose group, and in spite of the observed alterations in the genital tract, reproduction did not seem to be impaired.

In captive cheetahs fed soy protein containing commercial diet liver disease and impaired fertility was observed (Setchell et al., 1987). The liver function improved when the diet was substituted with a chicken diet.

Also coumestrol-containing diets can influence reproductive processes (Medlock et al., 1995; Whitten and Naftolin, 1992; Whitten et al., 1995). Whitten and Naftolin (1992) described the effects of a diet containing 0.01 % coumestrol on reproduction processes in female rats. Progesterone receptor induction was observed in the uterus, pituitary and hypothalamus-preoptic area, vaginal opening was accelerated and after chronic exposure vaginal cycling was irregular. In another study in rats, coumestrol suppressed estrogenic cycles in females and, dependent on time of treatment during lactation, persistent oestrus in females and changes in sexual behaviour of the male offspring developed (Whitten et al., 1995).

Neonatal mice proved also to be very susceptible for estrogenic compounds such as coumestrol, and showed long-term reproductive tract alterations after neonatal exposure (Burroughs, 1995).

Effects of clover phytoestrogens are described for cattle (Kallela et al., 1984) in Europe and in cattle and sheep in Australia and New Zealand (Adams, 1995). Sheep grazing on lucerne showed macroscopic changes of the genital tract due to the coumestrol-containing diet (Canetero et al., 1996).

For pigs however, only one reference on effects of dietary phytoestrogens has been found. Prepubertal gilts fed a 20 % soybean meal containing diet for 14 weeks showed a slight enlargement of the vulva after five weeks, but at slaughter no changes in the genital tract could be observed (Drane et al., 1981). This lack of data is in contrast to the amount of data on estrogenic effects of feed contaminated with the mycotoxin zearalenone, for which pigs appear very susceptible.

As compared to soybeans, the levels of phytoestrogens in peas, broad beans, lupines and quinoa are very low (10^3 - 10^5 lower than soybean). Since soybean meal is included in conventional pig feed without reported effects on fertility, it is not likely that the much lower levels of phytoestrogens from peas, broad beans and quinoa will be able to affect fertility negatively.

Considering the data from literature dietary phytoestrogens apart from estrogenic mycotoxins, do not appear to influence fertility in pigs.

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