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Metabolism of phytoestrogens in red clover and effects on sow reproduction

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Errata

- Concentration of equol in abstract should be expressed in $\mu\text{g mg}^{-1}$ creatinine.
- Blood was analyzed, however results not included in the presentation.



Background

- Legume forage crop silage is an alternative fiber-rich dietary supplement to enhance the welfare of feed-restricted sows during the gestation period
- Red clover (*Trifolium pratense*) contains phytoestrogens (PE), such as formononetin, biochanin A, daidzein and genistein.
- PE can interact with oestrogen receptors α and β , influencing estrogenic activity in the body and cause fertility problems and reproductive disorders, e.g. *Clover disease* in sheep.



Background

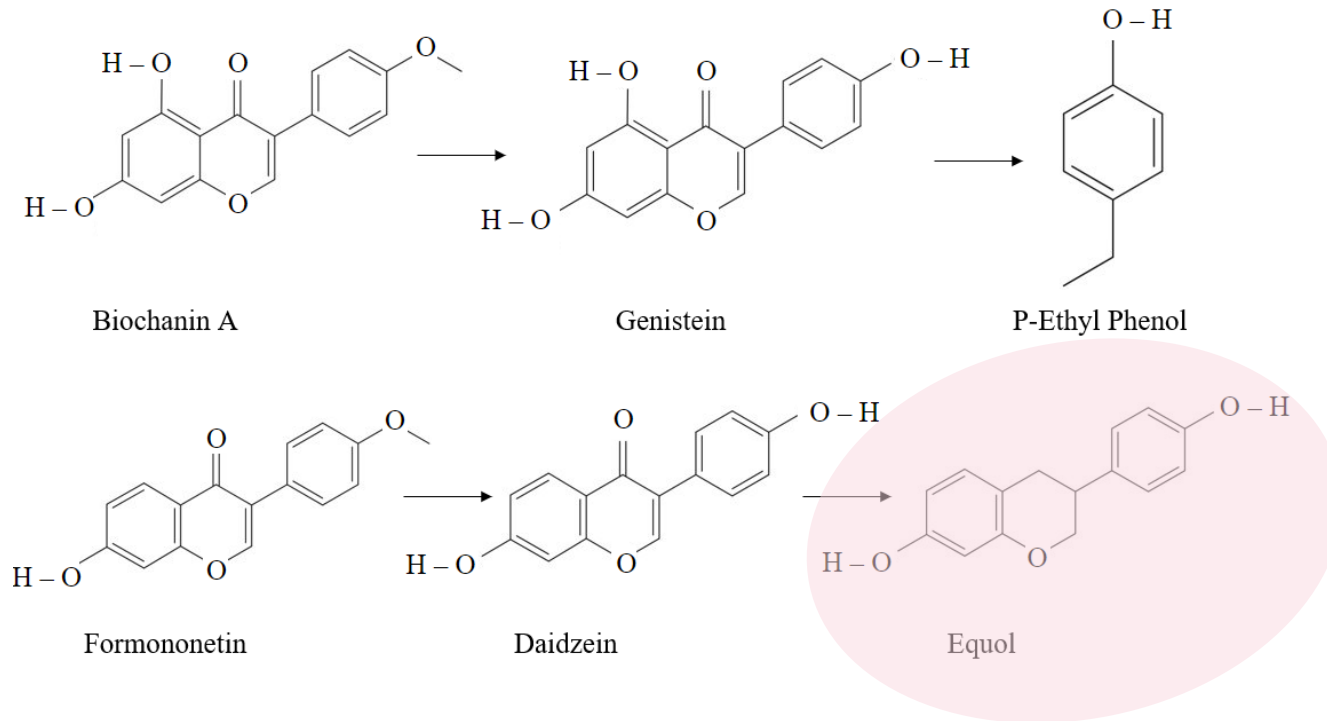


Figure 1. The chemical structure and major metabolic pathways of biochanin A and formononetin.

- Biochanin A and Formononetin key isoflavones in red clover.
- Demethylation by bacterial reactions in the rumen, distal intestine/colon.
- Equol - oestrogenic active metabolite.
- The capacity to produce equol varies among individuals.
- Only a few studies of the metabolism of PE in pigs.

Background

- After absorption by the intestinal epithelial cells, free form of PE can re-conjugate with glucuronic acid or sulphate groups to become more water-soluble and excreted in the urine
- Conjugation and elimination of these substances help reduce this risk of interaction with oestrogen receptors.
- Free form of PE can potentially pose a greater risk of affecting fertility and hormone balance.



Objective , aim and hypothesis

- **Objective:** To be able to give safe dietary recommendations about feeding red clover to sows.
- **Aim:** To better understand the dynamics of PE metabolism in sows fed red clover silage and the effects of PE on fertility and sow reproduction.
- **Hypothesis:**
 - Sows produce equol and that equol stabilizes over time.
 - Sows fed additional forage crop silage with red clover inclusion can tolerate the plant PE without negative effects on reproduction traits.



Study 1

- 8 gestating sows (gestation day 30-60) were fed red clover (*Trifolium pratense*) silage (RC) for 4w
- 8 gestating sows (gestation day 57-84) not fed red clover silage (control) for 4w.
- Feed, faeces and urine analyzed for PE content.
- Faecal and urinary PE and equol analyzed at w1, w2, w3, w4.

Study 2

- 23 sows studied during two reproduction cycles (r1, r2).
 - 11 fed PE rich red clover (*Trifolium pratense*) silage (**RCS**)
 - 12 fed white clover (*Trifolium repens* L.) silage with no PE (**WCS**).
- Feed, faeces, urine and blood analyzed for PE content.
- Faecal and urinary PE and equol analyzed at day 0, 30 and 60 (r1)
- Heat signs, teat characterization and udder filling registered at day 30, 60 and 90 after insemination in r1 and r2

Study 1

- **Pregnancy**
 - Group gestation pens on deep straw litter.
 - Individual feeding crates and *ad libitum* water access from a one-water nipple.
 - Feeding twice daily with a commercial pellet feed for dry sows, 100% feed ration, according to their nutrient requirements .
 - 4.3 kg (fresh) red clover silage, fed together with the commercial feed on the concrete floor in the feeding crates.
 - Silage constituted from a pure stand red clover ley.

Study 2

- **Breeding and pregnancy**
 - Same housing and feeding as study 1.
 - 3 and 1.5 kg (fresh) RCS or WCS fed together with the commercial feed on the concrete floor in the feeding crates.
- **Lactation**
 - Individual farrowing pens (6 m²),
 - Commercial feed for lactating sows, 100% feed ration.
 - 0.5-1 kg (fresh) additional RCS or WCS fed in racks.
 - RCS as in study 1. WCS from a mixed grass/white clover ley.





Extraction and separation of PE

- Feed and faeces were extracted by microwave-assisted extraction.
- Urine was extracted by solid phase extraction.
- Urine samples were analyzed for creatinine content using a urinary creatinine detection kit, to standardize the content of PE and equol.
- Separations were performed using UHPLC (Ultra-High-Performance-Liquid- Chromatography) and PE and equol were identified by their retention times and spectral characteristics at 260 and 280 nm.
- For determination of the total (free and conjugated) amount of PE, a solution of β -glucuronidase-sulfatase was added.
- Results processed in Empower and adjusted for the dilutions of the samples.



Registrations of reproduction parameters

- Over two reproduction cycles and three periods relative to the insemination date (30, 60 or 90 days), the sows were observed for reproductive traits.
- Udder and vulva characteristics, with a total of 120 observations (54 for RC fed sows and 66 for white clover fed sows).
- The frequency of observations within each treatment group was then evaluated using by descriptive analyses.

Main findings

- Total amount of PE in the red clover was $10.9 \text{ g kg}^{-1} \text{ DM}$
 - Highest conc. of formononetin and biochanin A (9.0 and $1.5 \text{ g kg}^{-1} \text{ DM}$).
 - Lower conc. of genistein and daidzein (0.2 and $0.1 \text{ g kg}^{-1} \text{ DM}$)
 - Daily PE intake of sows was 9.8 g
 - 8.1 g day^{-1} formononetin and 1.4 g day^{-1} biochanin A
- No PE and equol present in faeces and urine from sows in the C treatment.

- Higher concentrations of total amount of PE in urine than in faeces.
- PE in faeces and urine were to some extent affected by time.
- Faecal and total urinary equol increased and stabilized over time.
- Concentrations of free (unconjugated) PE and equol in urine were low or under detection level, except for daidzein.

Results Study 1

Concentrations of PE and equol in RC sow faeces (mg kg⁻¹ DM).

	Week					
	1	2	3	4	SEM	P-value
Biochanin A	11.7	10.3	9.7	10.0	0.6	0.100
Genistein	3.5 ^a	0.6 ^{ab}	0.3 ^b	0.3 ^b	0.7	0.002
Formononetin	5.3 ^a	17.6 ^b	4.0 ^a	0.0 ^a	3.8	0.001
Daidzein	12.7 ^a	19.6 ^{ab}	23.0 ^b	27.3 ^b	2.8	0.001
Equol	34.9 ^a	116.6 ^{ab}	274.3 ^{ab}	335.0 ^b	98.6	0.011

Concentrations of total (free and conjugated to glucuronic acid and sulphate) amounts of PE and equol in RC sow urine (µg mg⁻¹ creatinine).

	Week					
	1	2	3	4	SEM	P-value
Biochanin A	70.2 ^a	36.6 ^b	77.1 ^a	71.8 ^a	8.6	0.001
Genistein	28.7 ^a	16.9 ^b	29.2 ^a	26.6 ^a	3.5	0.001
Formononetin	222.3 ^a	105.3 ^b	227.4 ^a	207.2 ^a	20.8	0.001
Daidzein	12.8	11.0	14.5	13.1	1.8	0.360
Equol	119.1 ^a	153.3 ^{ab}	208.8 ^b	204.8 ^b	28.8	0.006

Concentrations of free (unconjugated) PE and equol in urine (µg mg⁻¹ creatinine):

- All PE (except for daidzein) = low or under detection level.
- Equol = under detection level
- Daidzein = similar level as the total amount.

Main findings

- Total amount of PE in **RCS** was 17.1 g kg⁻¹ DM
 - Highest conc. of formononetin and biochanin A (10.3 and 6.3 g kg⁻¹ DM).
 - Lower conc. of genistein and daidzein (0.4 and 0.2 g kg⁻¹ DM)
- Total amount of PE in **WCS** was 0.7 g kg⁻¹ DM
- Daily PE intake of **RCS** sows was 12.0 g
 - 7.2 g day⁻¹ formononetin and 1.4 g day⁻¹ biochanin A

- Lower amounts of PE and equol present in faeces and urine from sows in the WCS treatment.
- PE in faeces and urine were to some extent affected by time.
- Faecal and total urinary equol increased and some stabilized over time.
- Concentrations of free (unconjugated) PE and equol in urine were low or under detection level, except for daidzein.

Results Study 2 – PE metabolism

Concentrations of PE and equol in RCS and WCS **sow faeces**, at different days and interaction diet x day (mg kg⁻¹ DM).

	Diet			Day				P-value		
	RCS	WCS	SEM	0	30	60	SEM	Diet	Day	Diet x day
Biochanin A	12.5	8.92	0.73	5.8 ^a	11.1 ^b	15.3 ^b	1.18	0.002	0.001	0.018
Genistein	3.07	5.71	1.44	4.2	4.14	6.1	1.62	0.200	0.886	0.838
Formononetin	17.9	2.89	3.40	4.4 ^a	9.9 ^a	19.9 ^b	3.36	0.001	0.001	0.250
Daidzein	28.9	22.9	1.85	18.4 ^a	26.7 ^b	32.8 ^b	2.35	0.001	0.001	0.133
Equol	226.7	32.6	30.26	86.1 ^a	118.0 ^a	184.9 ^b	27.0	0.001	0.001	0.011

Different letters displayed in rows indicate statistically significant differences among the means

- Effect of diet – higher conc. in RCS, except for genistein.
- Effect of day – increase from day 0 to day 30 and 60 or from day 30 to 60, except for genistein.

Results Study 2 – PE metabolism

Concentrations of total (free and conjugated to glucuronic acid and sulphate) amounts of PE and equol in in RCS and WCS **sow urine**, at different days and interaction diet x day ($\mu\text{g mg}^{-1}$ creatinine).

	Diet			Day				P-value		
	RCS	WCS	SEM	0	30	60	SEM	Diet	Day	Diet x day
Biochanin A	18.4	2.28	2.51	1.75 ^a	13.7 ^b	15.6 ^b	2.93	0.001	0.002	0.037
Genistein	27.1	11.4	2.11	30.5 ^a	12.4 ^b	14.9 ^b	2.95	0.001	0.001	0.268
Formononetin	23.4	4.71	5.54	0 ^a	23.2 ^b	20.3 ^b	6.09	0.026	0.032	0.289
Daidzein	23.3	12.0	1.85	28.0 ^a	12.7 ^b	12.3 ^b	2.12	0.001	0.001	0.059
Equol	83.0	11.9	9.54	1.10 ^a	67.3 ^b	73.9 ^b	9.69	0.001	0.001	0.001

Different letters displayed in rows indicate statistically significant differences among the means

- Effect of diet – higher conc. in RCS.
- Effect of day – increase for biochanin A, formononetin and equol, decrease for genistein and daidzein from day 0 to day 30 and 60 .
- Concentrations stabilized over time.

Concentrations of free (unconjugated) PE and equol in urine ($\mu\text{g mg}^{-1}$ creatinine):

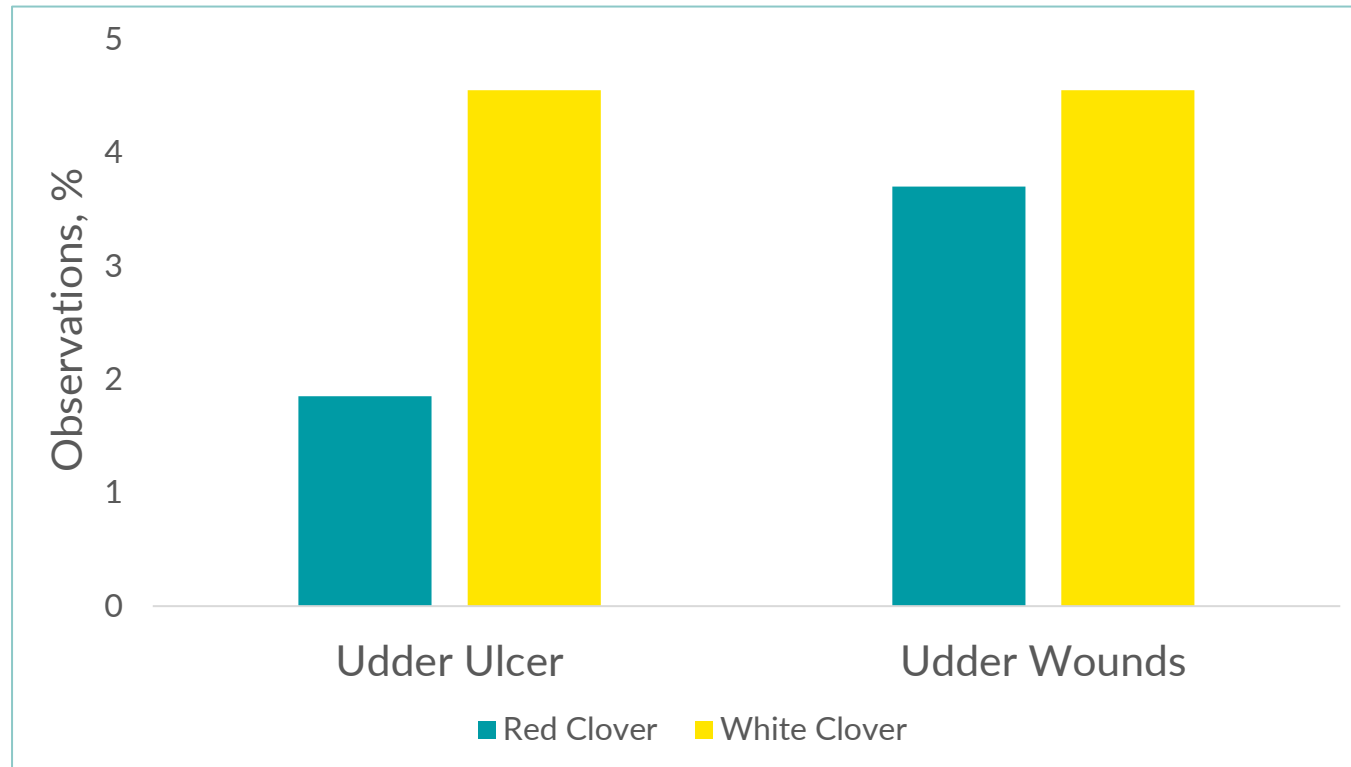
• All PE (except for daidzein) = low or under detection level.

• Equol = under detection level

Results Study 2 – Effect on reproduction traits

Udder redness, ulcer, wounds

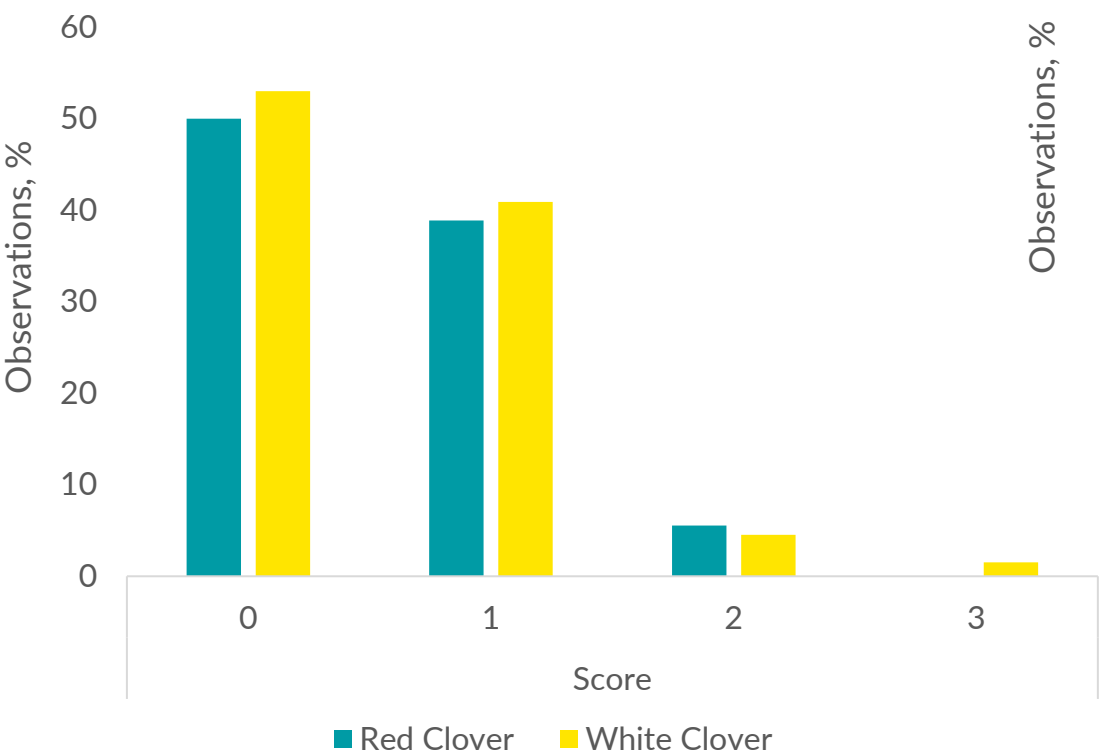
- No udder redness was identified.
- Observations of ulcers and wounds mainly found in reproduction cycle II



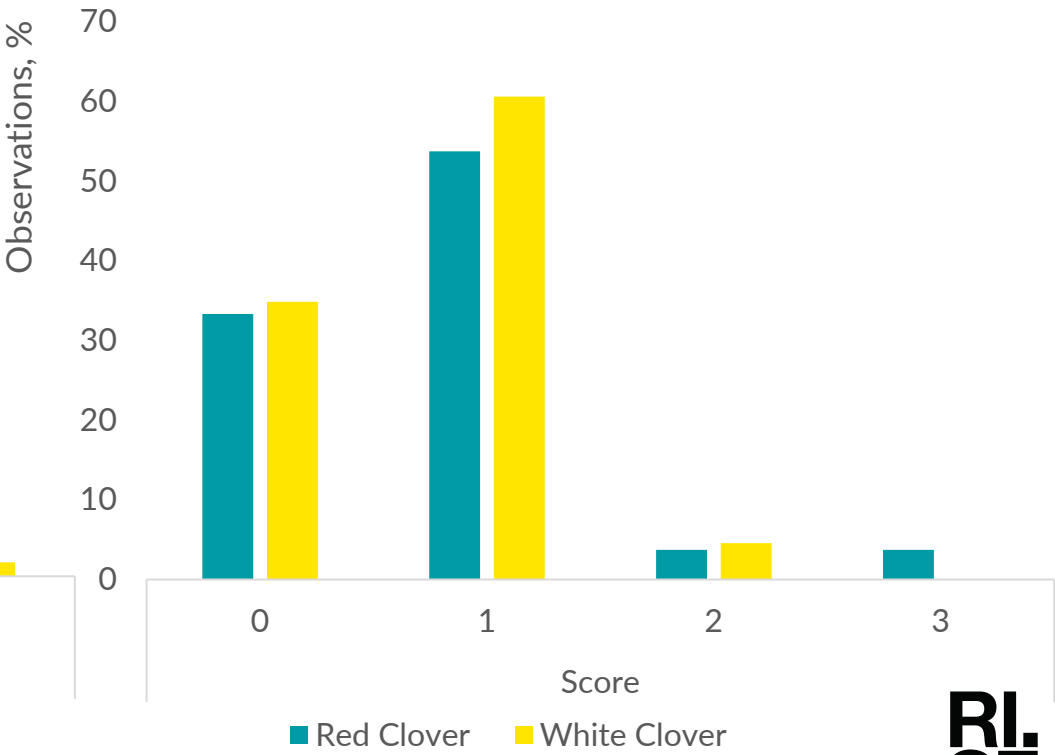
Results Study 2 – Effect on reproduction traits

Vulva swelling and redness

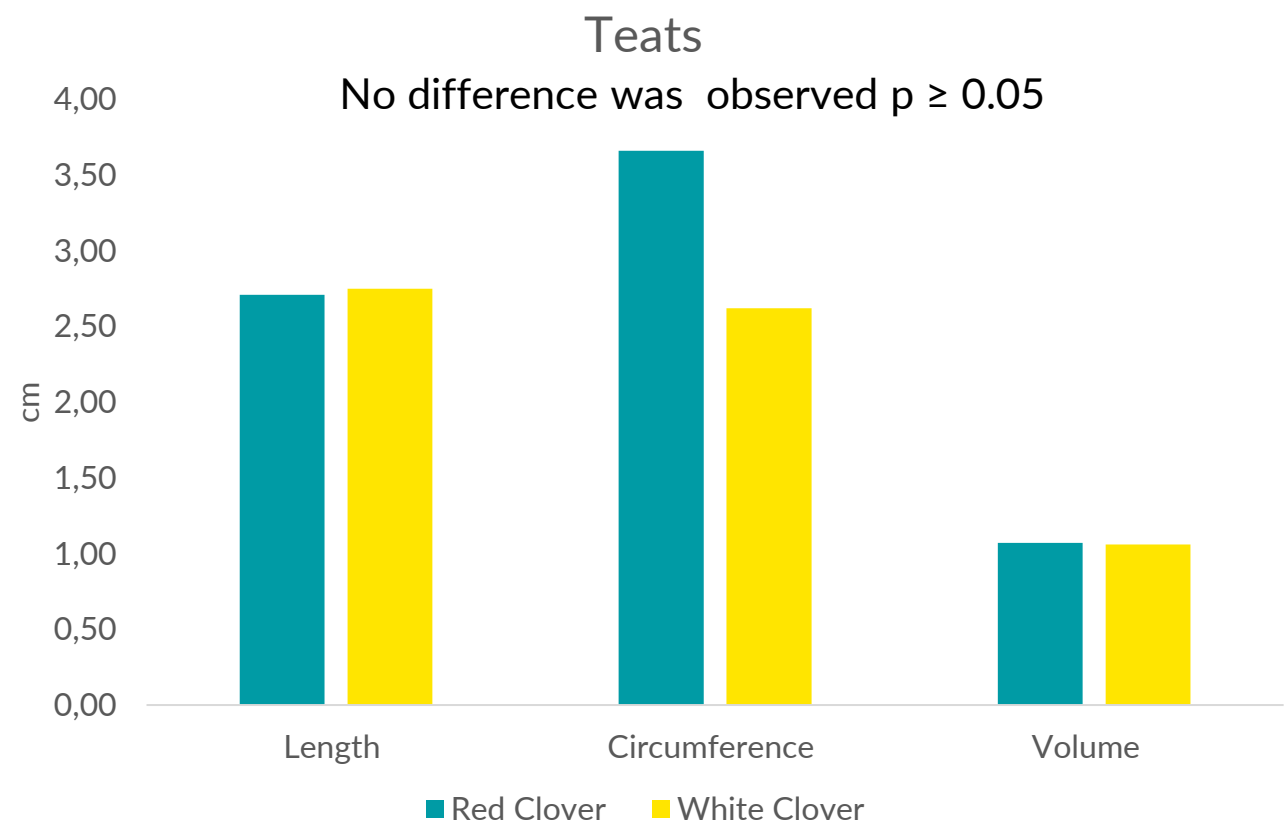
Vulva swelling



Vulva Redness



Results Study 2 – Effect on reproduction traits
Teats length, circumference and volume



Conclusions

- Sows fed additional red clover silage in the present studies efficiently metabolized formononetin and daidzein and produced equol.
- Major part of the excreted urinary PE and equol were conjugated to glucuronic acid and sulphate, supporting that sows have the capacity to eliminate equol.
 - Reduced risk of hormonal disturbances.
- No effect of RCS and WCS silage on reproduction cycle on reproduction traits ($P \geq 0.05$) and all sows had normal udder filling.
- More studies needed for dietary recommendations.



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