metabolism of phytoestrogens in dairy cows - effect of botanical composition of silages

omsetjing av planteøstrogen hjå mjølkekyr - effekt av botanisk samansetjing av surfôret

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master thesis 30 credits 2011

Forord

Denne masteroppgåva markerar avslutninga på fem fine år med studiar ved Universitetet for miljø- og biovitenskap, noko som er både godt og vemodig. Fem flotte og lærerike år har det vore, med fantastiske folk og eit inspirerande miljø!

Gjennom heile studiet var eg var klar på at masteroppgåva mi sku omhandla drøvtyggjarernæring, og då eg fekk moglegheit til å vera med på forsøk var eg snar med å sei ja. Forsøksarbeidet har vore veldig variert, kjekt og lærerikt. Eg har vore igjennom hausting, botanisering av eng og fôringsforsøk med det prøveuttaksarbeid og preparering som høyrer til. Analysar av planteøstrogen på Foulum og ikkje minst; arbeid med resultat og formidling av dei i masteroppgåva.

No, når arbeidet med masteroppgåva er slutt er det mange som skal takkast. Stor takk til mine vegleiarar Erling Thuen og Håvard Steinshamn for hjelp, støtte og vegleiing gjennom skriving av masteroppgåva. Tusen takk til Steffen Adler som har hatt meg med på forsøk, kome med resultat og svara på små og store spørsmål. Takk til Lis Sidelmann ved Det Jordbrugsvidenskabelige Fakultet, Foulum, for hjelp ved utføring av analysar, samt gode svar på spørsmål som dukka opp i etterkant. Ein særskild takk til Håvard som skubbar meg ut i utfordringar eg helst ikkje vil ha, men som eg er veldig glad for å få! ☺

Tusen takk til Anne, David, Eirik mamma og pappa for gjennomlesing og gode konstruktive tilbakemeldingar på oppgåva.

Tusen takk til Maria og Linn Hege for ei flott tid på lesesalen, gode faglege innspel og veldig mange ikkje fult så faglege!

Tusen takk til Jostein, for at du er den du er!

Institutt for Husdyr- og Akvakulturvitenskap

Ås, mai 2011

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Sammendrag

Omsetjing av isoflavona; biochanin A, genistein, formononetin, daidzein, equol og prunetin, lignana; matairesinol, secoisolariciresinol, enterodiol og enterolakton og coumestanet coumestrol er undersøkt. Studia har som mål å beskriva omsetjinga av planteøstrogen hjå mjølkekyr, og sjå på moglegheiter for å påverka innhaldet av planteøstrogen i mjølk. Fire kyr (Norsk Raudt Fe) var brukt i eit 4x4 latinsk kvadrat, med fire ulike surfôrtypar; raudkløver, artsrik eng, raigras og timotei. Konsentrasjonen av planteøstrogen vart målt i fôr, bladmage-fasar, gjødsel, urin og mjølk. Til analysar vart veskekromatografi (LC)-massedeteksjons ((MS)/MS) teknikken nytta (LC-(MS)/MS). Omsetjing i vom/nettmage og i tarmkanalen, samt overføringsgrad til mjølk er berekna.

Den høgaste konsentrasjonen av isoflavonar vart funne i raudkløver surfôr, og lågast i surfôr av timotei. For lignanar var det omvendt. Planteøstrogena vart i stor grad omsett i vomma/nettmagen. Omsetjinga av biochanin A og genistein var størst på raukløver-dietten og lågast på timotei-dietten. I omasum vart det funne ein større mengde av endogene lignanar; enterodiol og enterolakton, enn inntaket av secoisolariciresinol og mateiresinol. Dette indikera at det finnes andre og ukjende forløparar til endogene lignan i fôret. Isoflavonar og coumestrol følgde hovudsakeleg store partiklar ved passasje ut av nettmage, mens lignanar i større grad var fordelt mellom fasane. Skilnadar i konsentrasjon av planteøstrogen mellom diettar vart i stor grad redusert gjennom fordøyelses-kanalen, og for ein del planteøstrogen var det ingen effekt av diett på konsentrasjon i gjødsel. Konsentrasjonen av planteøstrogen i mjølk var aukande ved aukande konsentrasjon i fôret. Raudkløver-dietten hadde den høgaste konsentrasjonen av isoflavonar i mjølk og equol var dominerande. Det ser ut til at det er mogleg å påverka innhaldet i mjølk via fôring, men effekten er minkande ved aukande konsentrasjon i fôret.

Abstract

Metabolism of the isoflavones biochanin A, genistein, formononetin, daidzein, equol and prunetin, the lignans matairesinol, secoisolariciresinol, enterodiol and enterolactone and the coumestan coumestrol was investigated to study the metabolism of phytoestrogens in dairy cows, and the possibilities to influence the content in milk. Four Norwegian Red Cows where allocated in a 4x4 latin square with four different silages. Silages had different botanical composition; red clover, botanical diverse, perennial ryegrass and timothy. The concentration of phytoestrogens was measured in feeds, omasum phases, feces, urine and milk by using the liquid chromatography (LC)-mass spectrometry (MS)/MS technique. Metabolism in reticulo-rumen and in digestive tract as well as apparent recovery in milk was calculated.

Concentration of isoflavones was highest in red clover silage and lowest in timothy silage. For lignans it was the opposite. Phytoestrogens was extensively metabolized in reticulo-rumen. Biochanin A and genistein had the highest metabolism on the red clover diet and lowest on timothy. A number of unknown lignans was metabolized to the mammalian lignans enterodiol and enterolactone in the reticulo-rumen. When passing to omasum, isoflavones and coumestrol mainly followed large particles, while lignans was in greater extent evenly distributed between phases. Through the digestive system, differences in content of phytoestrogens between diets became smaller or disappeared. The concentration of isoflavones in milk was increasing with increasing intake. Red clover diet had the highest concentration of isoflavones in milk, with a dominance of equol. It appears that concentration of phytoestrogens in milk can be manipulated trough intake, but the effect is diminishing with increasing concentration in feeds.

Innholdsfortegnelse

1.0 Innleiing

Planteøstrogen er naturleg førekomande forbindingar med opphav i plantar. Ein kjemisk struktur liknande endogent østrogen, østradiol, gjer at planteøstrogen kan binda seg til østrogenreseptorar og kan dermed gje østrogeneffekt eller antiøstrogeneffekt på dyr og menneskjer (Côrtes et al. 2008; Sirtori et al. 2005; Tham et al. 1998). Østrogeneffekt av kløver vart først kjend i Australia tidleg på 1940 talet, der Bennetts et al.(1946) rapporterte at sauer som beita raudkløver fekk fertilitetsproblem (Cornwell et al. 2004; Cox & Braden 1974). I dag er interessa for planteøstrogen hovudsakeleg knytt til fordelaktig effekt på ei rekke sjukdommar, blant ulike hormonrelaterte kreftformar, hjarte og kar sjukdommar, plagar knytt til overgangsalder og osteoporose. Samtidig er det også stilt spørsmål om påverknad av østrogen kan ha uheldig effekt på spedbarnsutvikling (Cornwell et al. 2004; Mendez et al. 2002; Tham et al. 1998; Tuohy 2003; Zung et al. 2001).

I kroppen kan planteøstrogen bli omsett til endogene planteøstrogen, som equol, enterodiol og enterolakton. Endogene planteøstrogen har ein sterkare østrogeneffekt enn utgansstoffa dei er danna frå (Cornwell et al. 2004; Dickinson et al. 1988). Om lag 30-50 % av befolkninga har ein mikroflora i tarmen som har evne til og produsera equol (Frankenfeld 2011). Mjølk kan vera ei kjelde til meir verksamt endogent planteøstrogen for menneskjer, og det er difor interessant å undersøkja i kor stor grad ein kan påverka innhaldet i mjølk.

Denne masteroppgåva består av to delar. Første del er ein litteraturgjennomgang der det er sett på kjemisk klassifisering, innhald i fôr og mat, omsetjinga i kroppen og effekt av planteøstrogen. Andre del av oppgåva er eigne studiar av omsetjing av planteøstrogen i mjølkekyr. Formålet med dette studiet var å undersøkja korleis planteøstrogen blir omsett i fordøyelses-kanalen og sjå på mogligheitene for å påverka innhaldet i mjølk. Studiet er ein del prosjektet “ Potential improvement of the salutary effects of organic milk by forage species and by supplementation (PhytoMilk).” Andre del av oppgåva er skriven som ein vitskapeleg artikkel.

2.0 Teoridel

2.1 Kjemisk klassifisering av planteøstrogen

Planteøstrogen er syntetisert frå fenylpropanoidar og enkle fenolar (ringstrukturar) (Duncan et al. 2003; Setchell 1998). Fire ulike grupper med fenoliske strukturar blir betrakta som planteøstrogen; isoflavonoidar, stilbenar, lignanar og coumestanar (Tabell 1)(Cornwell et al. 2004).

Tabell 1. Kjemisk klassifisering av planteøstrogen (Cornwell et al. 2004; Heinonen 2006)

Gruppe	Undergruppe	Eksempel på planteøstrogen	Opphav
1. Isoflavonoidar	Isoflavonar	Genistein Daidzein Biochanin A Formononetin	Plantar. Særskild soya og kløver.
	Isoflavanonar	Dihydrodaidzein Dihydrogenistein	
	Isoflavanar	Equol	Fermentasjonsprodukt frå mikroorganismar.
	α-methyldeoxybenzoinar	O-demethylangolensin	
2. Stilbenar		Resveratol	Plantar. Særskild druer og peanøtter.
3. Lignanar		Matairesionol Secoisolariciresinol	Plantar. Særskild fiberhaldige.
		Enterolakton Enterodiol	Fermentasjonsprodukt frå mikroorganismar.
4. Coumestanar		Coumesterol	Plantar. Særskild kløver og spirer.

Det er påvist innhald av planteøstrogen i over 300 ulike plantearter, og 360 ulike isoflavonar var rapportert i 1994 (Heinonen 2006; Seested et al. 2000). Viktige planteøstrogen som kan påverka human og dyrehelse er isoflavonane genistein, daidzein, biochanin A og

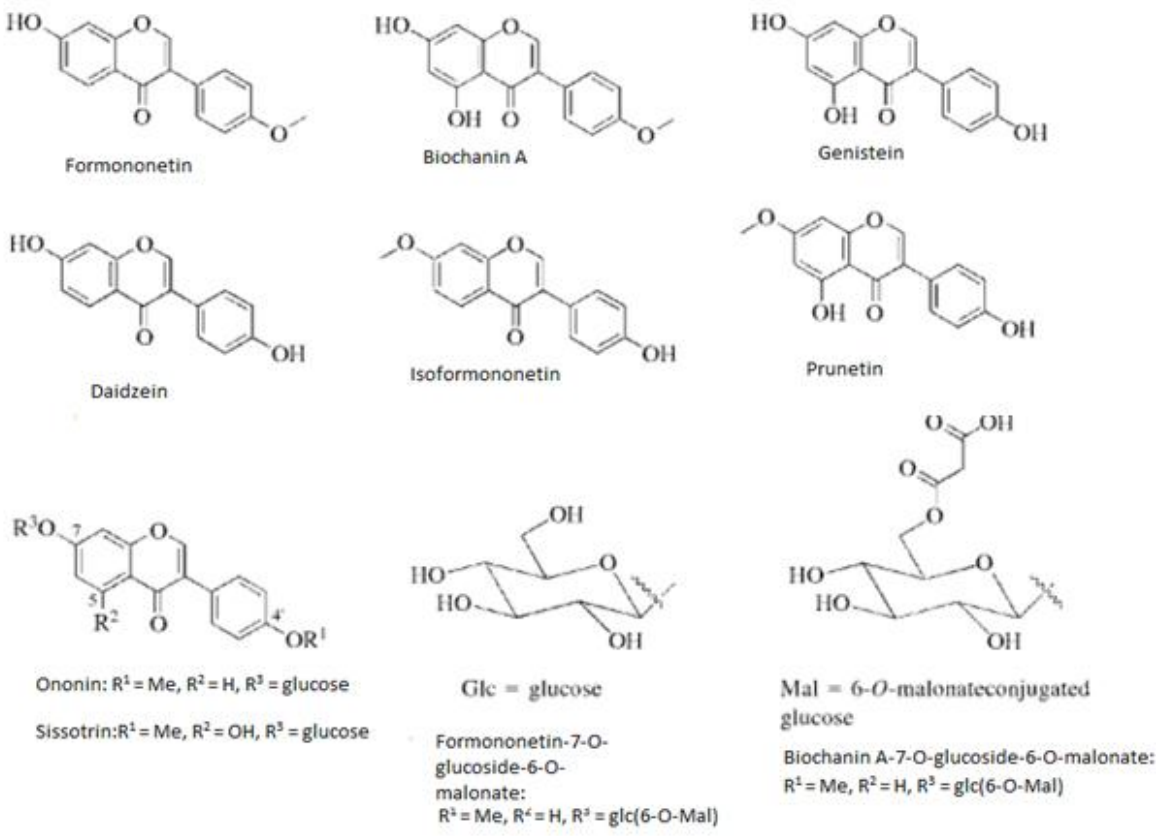
formononetin, coumestanet coumestrol og lignanane mateiresinol og secoisolariciresinol (Dixon 2004). Desse vil bli omtala vidare i oppgåva.

I plantar kan planteøstrogen ha form som eit glykosid (genistin, daidzin) eller som eit aglykon (genistein, daidzein) (Cassidy et al. 2000). Eit glykosid er eit stoff danna frå ein reaksjon mellom ei hydroksylgruppe (-OH) i eit karbohydrat, konjugert med ei hydroksylgruppe i ein anna organisk forbinding. Eit aglykon er komponenten som er igjen i eit glykosid etter at karbohydratet er spalta av.

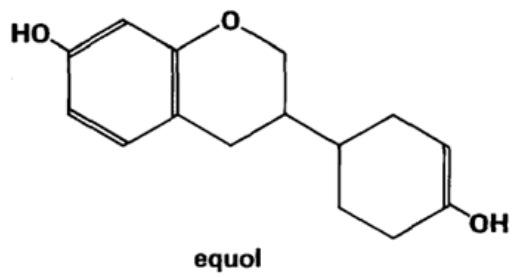
2.1.1 Isoflavonoidar

Isoflavonoidar blir inndelt i fire ulike undergrupper, isoflavonar, isoflavanonar, isoflavanar og α-methyldeoxybenzoinar (Heinonen 2006). Tabell 1 viser denne inndelinga. Isoflavonar og isoflavanar vil i hovudsak bli omtala vidare.

Isoflavonar er ei stor gruppe av isoflavonoidar (Mazur & Adlercreutz 1998). Over 60 ulike isoflavonar, bestående av ein 1,2-diarylpropan struktur, er identifisert og ein del viktige er vist i Figur 1 (Cassidy et al. 2000; Taponen et al. 2010). Genistein, daidzein, biochanin A og formononetin er dei mest undersøkte og viktigaste knytt til østrogeneffekt (Mazur & Adlercreutz 1998). Isoflavonar dannar eit glykosid med eit glukosemolekyl i plantar (Bingham et al. 1998).



Figur 1. Viktige konjugerte og ikkje konjugerte isoflavonoidar. Ononitin, Sissotrin, Formononetin-7-*O*-glucoside-6-*O*-malonate og Biochanin A-7-*O*-glucoside-6-*O*-malonate er eksempel på glykosid (Taponen et al. 2010).



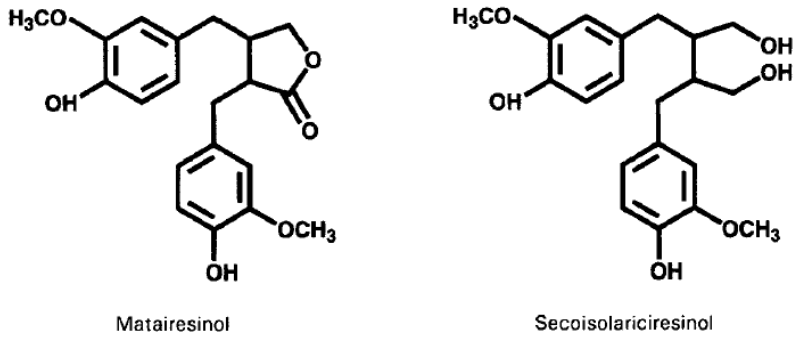
Figur 2. Equol (Adams 1995).

Isoflavanet equol (Figur 2) er det viktigaste produktet med østrogeneffekt av omsetjinga av isoflavonar i fordøyelseskanalen. Det finnes to ulike stereoisomeriske former av equol; *S*-

equol og *R*-equol, der *S*-equol blir produsert av mikroorganismar i tarmen på menneskjer og i vomma hjå drøvtyggjarar (Taponen et al. 2010).

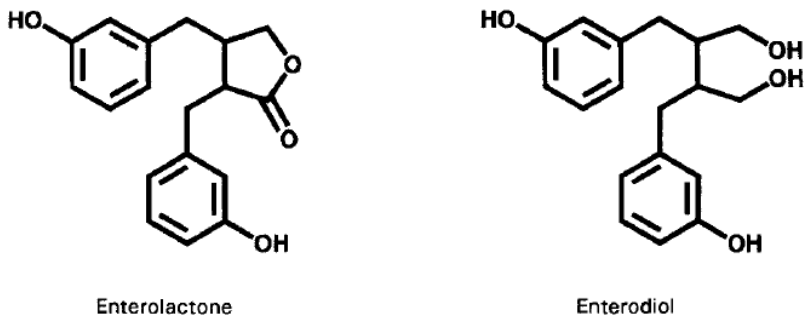
2.1.2 Lignanar

Lignanar består av ein 2,3-dibenzylbutan struktur, og er ein bestanddel i danninga av lignin (Tham et al. 1998). Det er to hovudgrupper av lignanar, secoisolariciresinol og matairesinol (Figur 3). Begge grupper er i form av eit glykosid i plantematerialet (Magee & Rowland 2004).



Figur 3. Lignanar i fôr (Bingham et al. 1998).

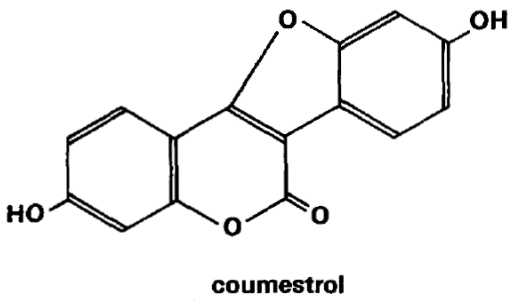
Secoisolariciresinol blir omsett til enterodiol i fordøyelseskanalen, som vidare kan bli omsett til enterolakton (Figur 4). Matairesinol blir omsett direkte til enterolakton (Kurzer & Zu 1997). Dei endogene lignana enterodiol og enterolakton liknar strukturelt på østradiol og kan gje østrogeneffekt (Bingham et al. 1998).



Figur 4. Enterolakton og enterodiol (Bingham et al. 1998).

2.1.2 Coumestaner

Coumestaner er ei stor gruppe forbindingar, der coumestrol (Figur 5) er det mest studerte. Coumestrol og 4´metoksycoumestrol er vist å gje østrogeneffekt (Cornwell et al. 2004; Magee & Rowland 2004). Coumestrol har ein sterkare østrogeneffekt enn isoflavonoidar og lignanar, då coumestrol blir absorbert og har østrogeneffekt utan omsetjing. Den har og meir liknande struktur som østradiol enn det isoflavanar har (Seested et al. 2000).



Figur 5. Coumestrol (Adams 1995).

2.2 Innhald i fôr og mat

I fôr til drøvtyggjarar finn ein planteøstrogen hovudsakeleg i soya *(Glycine max)*, jordkløver (*Trifolium subterraneum*), raudkløver (*Trifolium pratense*) og luserne (*Medicago sativa*). Kløver og soya inneheldt i hovudsak isoflavonar, luserne inneheldt i hovudsak coumestanar (Taponen et al. 2010). Innhaldet av isoflavonar i raudkløver er mellom 0,5-2,5 % av tørrstoffet (TS), der formononetin kan utgjera opptil 50 % (Seested et al. 2000). Raudkløver-blad inneheldt den største mengda isoflavonar, etterfølgd av stilk og blomsterstand, men det er signifikant variasjon mellom sortar (Adams 1995). I studie av innhald av isoflavonar i raudkløver fann Booth et al.(2006) at plantedelar over bakken inneheldt (i % av TS) 0,013-0,033 % daidzein, 0,014-0,068 % genistein, 0,21-0,59 % formononetin og 0,07-0,33 % biochanin A. Blomster hovuda inneheldt 0,0025-0,0079 %, 0,018-0,019 %, 0,047-0,12 % og 0,070-0,14 % av dei respektive planteøstrogena. Kvitkløver og luserne inneheldt 10-20 % så mykje planteøstrogen som i raudkløver, der isoflavonar var > 1 % av tørrstoffet, og innhaldet av formononetin var om lag 0,2 g per kg tørrstoff (Seested et al. 2000).

Lignanar finn ein i hovudsak i celleveggen i heile korn, belgvekstar, grønsaker og frø, der linfrø har eit særs høgt innhald (Petit et al. 2009; Tham et al. 1998).

Luserne har eit innhald på 0,005-0,030 g coumestan per kg tørrstoff. Kvitkløver kan produsera coumestan dersom den er angrepet av sjukdom på blada (Adams 1995; Seested et al. 2000). Dersom kvitkløver er angrepet av soppsjukdom kan coumestaninnhaldet bli 0,2 g per kg tørrstoff, og innhaldet av formononetin kan bli fordobla (Seested et al. 2000). Coumestrol blir i hovudsak funne i kløver og spirer av soyabønner og grøne bønner (Magee & Rowland 2004).

Tabell 2. Innhald av planteøstrogen i vegetabilske mat og fôrvarer (Mazur & Adlercreutz 1998)

	µg/100 g tørrvekt			
vare	daidzein	genistein	secoisolariciresinol	matairesinol
kveite, heilkorn			33	3
kveite, kli	4	7	110	
havre, mjøl			13	
havre, kli			24	155
bygg, heilkorn	14	8	58	
bygg, kli	6	16	63	
rug, heilkorn mjøl			47	65
rug, kli			132	167
soyabønne	10 500 - 56 000	26 800 - 84 100	13 - 273	spor av
ert	4 - 11	0 - 23	3 - 13	0 - spor av
linfrø			369 900	1 087
solsikkefrø	8	14	610	
blåbær			835	
bringebær		spor av	139	
eple	12		spor av	
banan			10	
hodekål	spor av	spor av	33	spor av
brokkoli	6	8	414	23
løk			83	8
gulerot			370	spor av
potet, utan skal			10	6

Tabell 2 viser at hovudtyngda av planteøstrogen i korn, oljefrø, bær og grønsaker er lignanet secoisolariciresinol. Soya inneheldt store mengder isoflavonar.

Tabell 3. Innhald av planteøstrogen i animalske matvarer, samt soyamjølk (Kuhnle et al. 2008)

Vare[1]	µg/100 g råvekt						
	planteøstrogen	Isoflavonar[2]	Lignanar[3]	coumestrol	equol	enterolacton	enterodiol
heilmjølk	12	6	1	<1	1	4	
lettmjølk	8	4	1	<1	1	3	
skummamjølk	20	14	1	<1	1	3	
geitemjølk, heil	5	1	1	<1	3	1	
soyamjølk, usøta	6 028	6 018	9	1			
iskrem, dessert	15	2	6	1	2	5	
ost, Camembert	29	3	7	<1	4	15	
ost, feta (sau -og geitemjølk)	12	4	<1	<1	2	5	
ost, Parmesan	27	6	5	1	4	11	
ost, cottage cheese	11	2	2	<1	1	7	
egg, "barn-kept hens," eggekvite	5	2	2	<1	<1	<1	<1
egg, "barn-kept hens," plomme	47	31	6	<1	8	2	<1
egg, "barn-kept hens"	18	8	2		4	3	1
egg, frittgåande høner	11	6	3	<1	1	1	
storfekjøtt, steikt, feit	19	3	16	1			
storfekjøtt, steikt, magert	7	1	6	<1			
kylling-bryst, steikt	6	4	2	<1			
lam, steikt, feit	10	5	4	1			
lam, steikt, magert	5	1	4	<1		<1	
svin, steikt, feit	8	3	5	1			
svin, steikt, magert	4	1	3	<1			
laks	4	3	1				
torsk, i mikrobølgjeomn	2	1	1				
blåskjel	9	7	2				
reker, frosne	8	3	4	1			
tunfisk, i saltlake på boks	6	5	<1	<1			

[1] Mjølkeprodukt er frå kumjølk med mindre noko anna er oppgitt.
[2] Isoflavonar er sum av biochanin A, daidzein, formononetin, genistein og glycitein.
[3] Lignanar er sum av secoisolariciresinol, matairesinol og shonanin.

Tabell 3 viser at innhaldet av planteøstrogen er særs høgt i soyamjølk, der hovudtyngda er isoflavonar. I ost er innhaldet av alle planteøstrogen høgare enn for mjølk, samt at ein i både ost og mjølk finn omsetjingsprodukt; equol og enterolakton. Eggeplomme inneheld meir

planteøstrogen enn eggekvite og isoflavonar er det dominerande planteøstrogenet. Feitt kjøtt inneheldt meir planteøstrogen enn magert kjøtt.

2.2.1 Faktorar som påverkar innhald i plantar og animalske produkt

Hovudkjelda til planteøstrogen for drøvtyggjarar i Sverige og Finland er raudkløver, (Lundh 1995; Sarelli et al. 2003), og det er rimeleg å anta at dette også gjeld for norske tilhøve. Sarelli et al. (2003) har vist i ein studie frå Finland at innhaldet av planteøstrogen i surfôr av raudkløver går opp 1,5 g/kg TS (15 %) frå før til etter ensilering. For surfôr hausta ved knoppdanning på raudkløver, var det ein nedgang i innhaldet av planteøstrogen ved bruk av maursyre som ensileringsmiddel framfor ein inokulant. For surfôr hausta ved raudkløver-bløming vart ikkje det vist noko effekt av ensileringsmiddel. Innhaldet av planteøstrogen vart redusert ved aukande alder på plantematerialet ved hausting og ved aukande grad av fortørke. Tørke til høy kan gje ein reduksjon i innhald av planteøstrogen oppimot 70 % (Seested et al. 2000).

Isoflavonet formononetin er hovudkjelda til planteøstrogen i kløver. Produksjonen av formononetin er genetisk bestemt, men ser ut til å bli påverka i noko grad av ytre faktorar: plantematerialet inneheldt mest planteøstrogen på våren, og innhaldet går ned ved bløming. I blømingsfasen kan innhaldet gå ned 1 % på 14 dagar. Dersom planteveksten er satt tilbake av næringsmangel, soppangrep eller frost i vekstperioden, kan innhaldet av formononetin auke oppimot 100 % (Adams 1995; Kallela et al. 1987; Seested et al. 2000). I kløver finn ein daidzein ved byrjande av-bløming, ikkje elles (Seested et al. 2000).

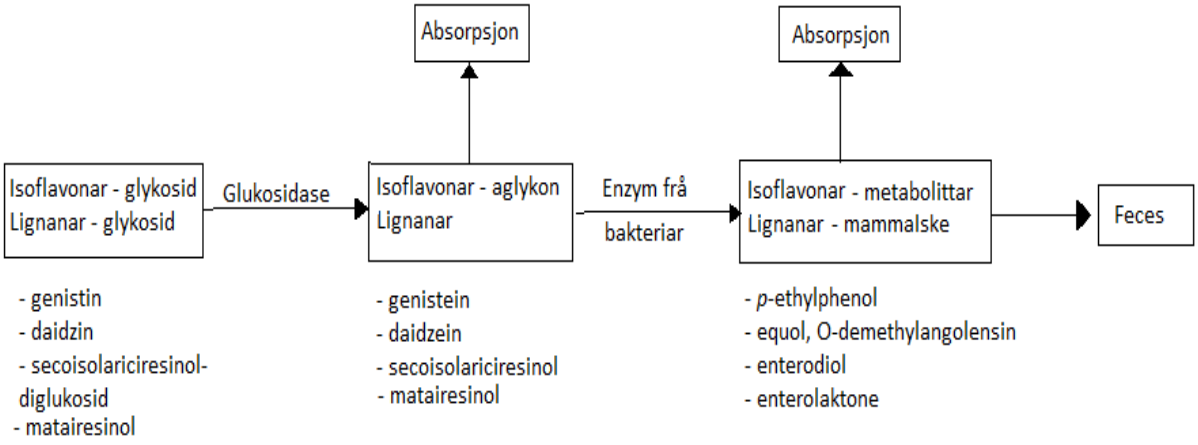
Innhaldet i animalske produkt er hovudsakeleg undersøkt i mjølk. Fleire ulike studiar har sett på moglegheit for å påverka innhaldet av planteøstrogen i mjølk ved ulik botanisk samansetnad på fôret. Purup et al. (2005), Hoikkala et al. (2007) og Antignac et al. (2004) har alle samanlikna økologisk produsert mjølk med konvensjonell og funne at innhaldet av isoflavonar er høgare i økologisk mjølk. Ein årsak til dette kan vera eit større innhald av belgvekstar i økologisk fôr til mjølkekyr (Steinshamn et al. 2008). Fôringsforsøk med ulike surfôrtypar viser at det er til ein viss grad mogleg å auka innhaldet av planteøstrogen i mjølk ved å auka innhaldet i fôr (Andersen et al. 2009; Steinshamn et al. 2008). I begge forsøka er det berekna kor stor del av inntaket ein finn igjen i mjølk, og det er vist at ved aukande konsentrasjon av planteøstrogen i fôret blir ein mindre del i % overført til mjølk. Steinshamn et al. (2008) fant 1,2 µg/mg formononetin, daidzein og equol i mjølk ved eit inntak av

formononetin og daidzein på 2,25 g/dag. Ved eit inntak på 34,7 g/dag fann dei 0,24 µg/mg i mjølk.

Effekt av formononetin på lam beitande på kløver er undersøkt, og viser at konsentrasjonen av equol i kjøttet ikkje vart påverka av inntaket av formononetin (Moorby et al. 2004).

2.3 Omsetjing av planteøstrogen

I plantar er planteøstrogena bunde til eit glukosemolekyl, og dannar eit glykosid. Den bundne forma er inaktiv, og gjev ingen østrogeneffekt før glukosemolekylet er spalta av (Bingham et al. 1998). Omsetjing i fordøyelseskanalen er vist i Figur 6. Hjå menneskje blir planteøstrogen hovudsakeleg omsett og absorbert i tjukktarmen, mens for drøvtyggjarar skjer omsettinga og absorpsjon både i vom og tjukktarm (Saarinen et al. 2002). Glykosida kan bli dekonjugert til aglykon ved hjelp av saltsyre eller *β*-glukosidase frå fôr eller mikroorganismar. Aglykonar blir vidare omsett til hormonliknande komponentar av mikrofloraen i tarmen. Lignanar kan bli omdanna til endogene lignaner; enterodiol og enterolakton, mens isoflavonar kan bli omdanna til O-demethylangolensin og equol (Cassidy et al. 2000; Duncan et al. 2003).



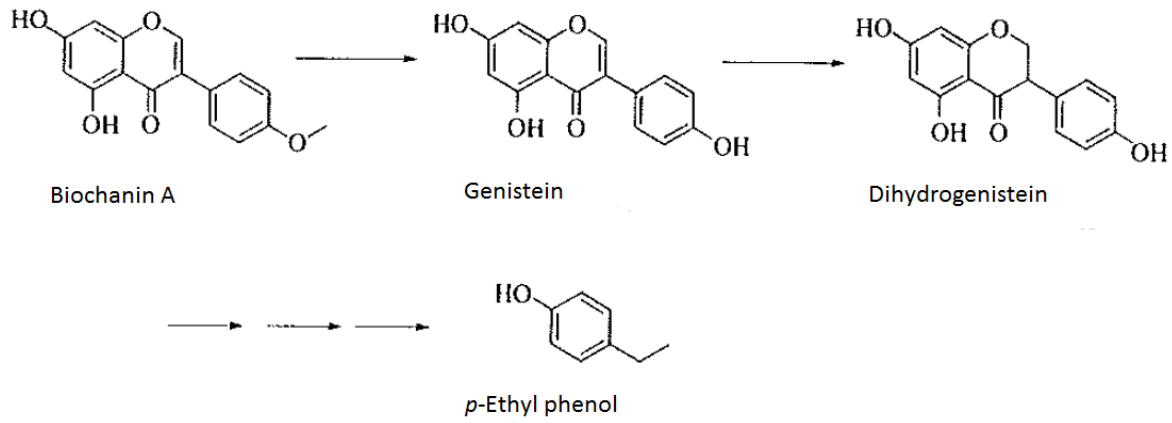
Figur 6. Omsetjing av planteøstrogen i fordøyelses-kanalen. Planteøstrogen kan bli absorbert som aglykon, som metabolittar eller utskild i feces i opphavleg eller omsett form (Duncan et al. 2003; Saarinen et al. 2002).

2.3.1 Isoflavonar

Omsetjing av biochanin A, genistein, formononetin og daidzein er særskild studert på sau (Mazur & Adlercreutz 1998). I hovudsak blir isoflavonar omsett og absorbert i vomma.

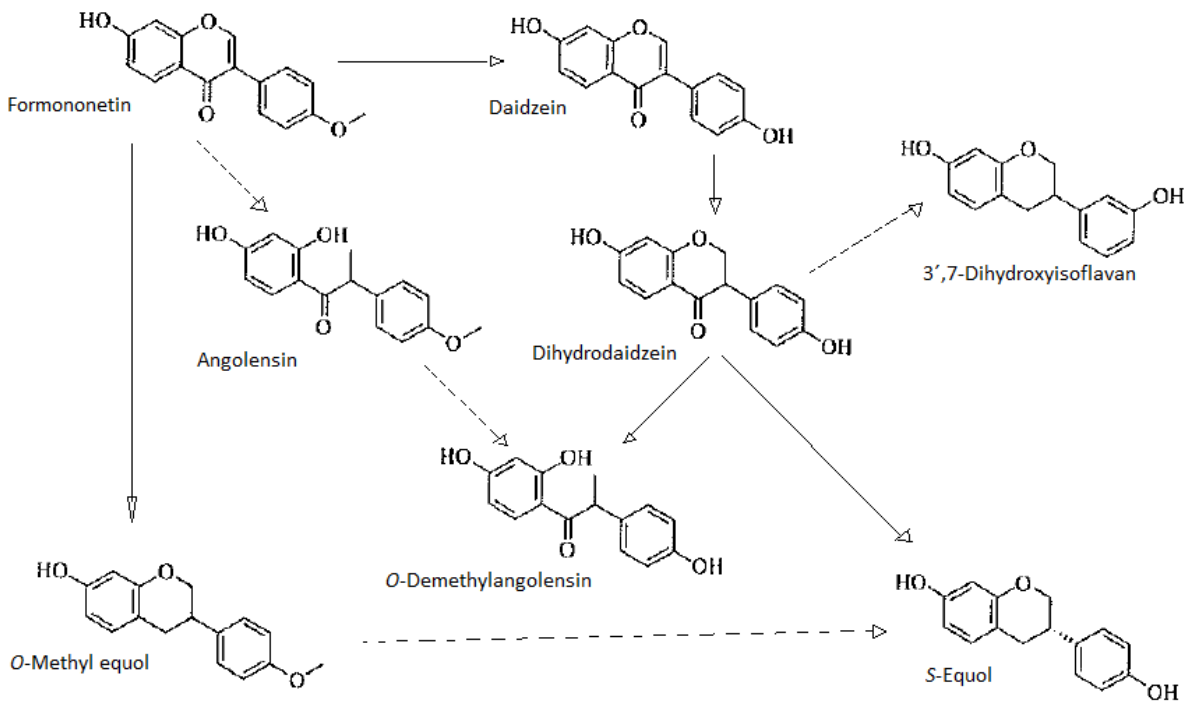
Mindre enn 1 % av inntaket av isoflavonar blir absorbert og utskild intakte (Taponen et al. 2010).

Biochanin A blir demetylert til genistein og vidare omdanna til *para*-ethyl-phenol og andre organiske syrer utan østrogeneffekt, Figur 7 (Andersen et al. 2009; Lundh 1995). Vom-floraen vil over tid bli stimulert til meir effektiv nedbryting av biochanin A og genistein, og etter 6-10 dagar vil alt bli fullstendig nedbrote, og ikkje gje noko østrogeneffekt. Dette skuldast truleg at vom-floraen endrar seg ved påverknad av planteøstrogen (Seested et al. 2000).



Figur 7. Metabolisme av biochanin A i vom (Taponen et al. 2010).

Formononetin blir redusert til equol enten via demetylering til daidzein eller *O*-demethylangolensin (Figur 8). Studiar gjort på sau viser at om lag 70 % av daidzein blir omdanna til equol, og 5-20 % blir omdanna til *O*-demethylangolensin (Cassidy et al. 2000). Sjølv om dei fleste studiar på metabolisme av isoflavonar er gjort på sau, ser det ut til metabolismen hjå storfe liknar kvalitativt på metabolismen hjå sau (Taponen et al. 2010).

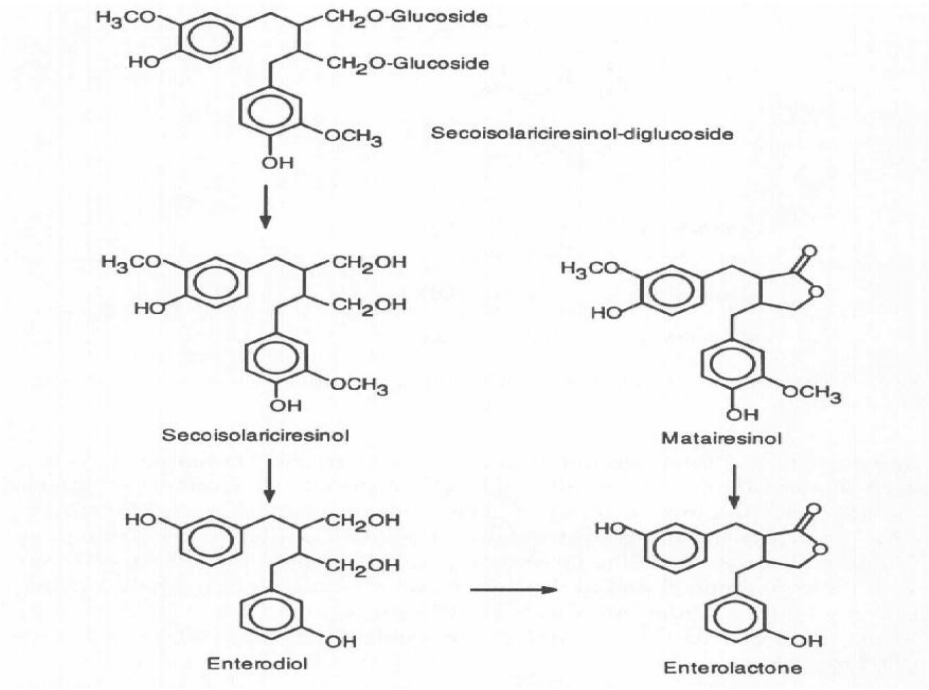


Figur 8. Omsetjing av formononetin hjå drøvtyggjarar (Taponen et al. 2010).

Formononetin, methylequol, daidzein, dihydrodaidzein, *O*-demethylangolensin, genistein og 3´,7-dihydroxyisoflavan er funne i urin frå menneskjer. Daidzein, equol, genistein og *O*-demethylangolensin er funne i blod og feces. Equol og *O*-demethylangolensin blir truleg hjå menneskjer danna ved same mekanismar som for sau, men av mikrobiell aktivitet i tarmen (Mazur & Adlercreutz 1998). Daidzein blir i tjukktarmen hjå menneskjer omdanna til equol eller til O-demethylangolensin og genistein kan bli omdanna til p-ethyl phenol. Daidzein, genistein, equol og O-demethylangolensin er dei vanlegaste planteøstrogena ein finn igjen i urin hjå menneskjer og dyr, men omdanning i tarmen er avhengig av mikrofloraen som er til stades (Tham et al. 1998). Ein del menneskjer har ikkje evne til å danna equol. Dermed treng ikkje equol funne i feces frå menneskjer ha blitt danna i tarmen, men kan stamma frå inntak av mjølkeprodukt eller kjøtt som inneheldt equol (Mazur & Adlercreutz 1998). Ein reknar med at om lag 20-35 % av den vaksne populasjonen av menneskjer kan få danna equol i tarmen (Taponen et al. 2010).

2.3.2 Lignanar

Forløparen secoisolariciresinol- diglukosid, som finnes i plantar, blir omdanna til secoisolariciresinol og vidare omdanna, saman med matairesinol (frå plantar), til endogene lignanar ved mikrobiell fermentering (Figur 9). Under fermentering blir glukose og metylgruppa spalta av, og difenolar, enterolakton og enterodiol blir danna (Bingham et al. 1998; Saarinen et al. 2002). Enterodiol kan også vidare bli omdanna til enterolakton i tarmen (Côrtes et al. 2008; Magee & Rowland 2004). For drøvtyggjarar skjer fermenteringa hovudsakeleg i vomma (Zhou et al. 2008).



Figur 9. Omsetjing av lignanar (Kurzer & Zu 1997).

2.3.3 Coumestanar

Coumestrol blir ikkje omsett i vomma, men blir absorbert over vom-veggen, og er biologisk aktiv i opphavleg form (Seested et al. 2000).

2.3.4 Intermediær omsetnad

Både aglykon og endelege omsetjingsprodukt av den mikrobielle fermenteringa kan bli absorbert. I kroppen blir forbindingane rekonjugert til glykosid, og utskild i urin, feces og

mjølk (Tham et al. 1998). Mikroorganismar reduserer og demetylerer planteøstrogen, mens konjugering truleg finn stad i epitelet i vom, nettmage, bladmage og tynntam.

Analysar av isoflavonar i blod frå sau og storfe viser at konsentrasjonen av isoflavonoidar ser ut til å vere på same nivå både for storfe og sau. Estimert mengde ikkje konjugert equol av total mengde equol er om lag 5 % for storfe og 1 % for sau (Taponen et al. 2010). Planteøstrogena blir så ført via portåra til levra, der ikkje konjugerte forbindingar blir konjugert med glykoronsyre eller sulfat. 5-15 % av isoflavonar blir konjugert med sulfat, resten med glykoronsyre. Forbindingar konjugert med sulfat er biologisk aktive, lik ikkje konjugerte forbindingar (Seested et al. 2000). Coumestrol blir i mindre grad konjugert i hepatisk og ekstrahepatisk vev enn isoflavonar og lignanar, og 20 -40 % sirkulerer fritt i blodet (Seested et al. 2000).

Konjugerte endogene lignanar kan bli utskild tilbake i tarmen via gallegangen. Slike lignanar blir i liten grad absorbert på ny i tarmen, men blir dekonjugert på ny ved hjelp av mikrobielt enzym (β-glykoronidase) og absorbert. Noko av enterolakton og enterodiol blir utskild via nyrene og noko går ut av dyret via feces i ukonjugert form (Gagnon et al. 2009a; Tham et al. 1998) I eit forsøk med rotter vart det gitt radioaktivt merka secoisolariciresinol, både som ein einskild dose og over tid. Prøvar vart tatt av vev, blod og tarminnhald. Det høgaste innhaldet av lignan fann ein etter 12 timar. Etter 48 timar var 80 % av tilført lignan funne igjen i urin og feces. Metabolittar såg ut til å hopa seg opp i lever, nyrer, tarmvev og livmor. Ved tilførsel over tid gjekk nivået i feces ned, men auka i lever og feittvev (Cassidy et al. 2000).

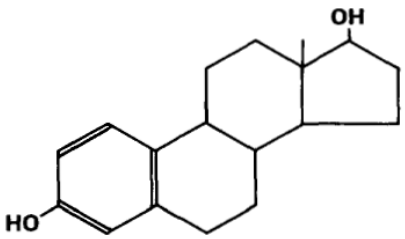
Planteøstrogen blir i hovudsak utskilde i urinen, men også i mjølk (Kuhnle et al. 2008). Utskiljing av planteøstrogen hjå drøvtyggjarar er ikkje mykje undersøkt, men det ser ut til at 70-80 % av isoflavonoidar blir utskild via urin. Isoflavonar blir også i noko grad utskild i mjølk, der equol er den dominerande. Innhaldet av equol variera, der det høgaste innhaldet er funne i økologisk mjølk og mjølk frå kyr fora med raudkløver. Konsentrasjonen av equol i mjølk ser ut til å vera om lag ein tiandedel av konsentrasjonen i blod (Taponen et al. 2010).

Omsetjing av isoflavonar hjå menneskjer kan bli påverka av ulike faktorar. I forsøk der det vart gitt 45 mg/dag i ein månad, viser at det er betydeleg individuell skilnad i metabolsk respons på lik mengde tilførte isoflavonar. Utskiljing av isoflavonar i urin varierte frå 1 til 17 µg/dag på kontrolldietten, og auka med mellom 0,4 til 8 mg/dag på forsøksdietten. Det var også individuelle skilnadar i innhald av equol i urinen, berre 2 av 6 testpersonar skilde ut equol, resten skilde ut daidzein og genistein. Ein manglande evne til å produsera equol kan

skuldast at mikroorganismane i tarmen har ein manglande evne til å produsera enzymet som er naudsynt for å danna equol. Studiar viser også at eit høgt inntak av karbohydrata i kosten aukar omdanninga av lignan og isoflavonar (Cassidy et al. 2000).

2.4 Effekt av planteøstrogen

Østrogen er ei gruppe steroidhormon, der østradiol er det viktigaste østrogenet. Steroida verkar ved og binda seg til reseptor-protein i cytocol og danna eit steroid-protein kompleks. Dette komplekset bitt seg til spesifikke DNA seter, kalla hormonresponsive elemnet, og påverkar transkripsjonen av genar i området. Dei viktigaste funksjonane til østrogen er å stimulera follikelvekst, påverka åtferd knytt til brunst, førebu kjønnsorgan på paring og transport av sæd, skapa gode veksttilhøve for embryo i livmora, bidra til vekst og utvikling av bryst- jurvev og førebu livmor til fødsel.



Figur 10. Endogent østrogen, østradiol 17-β (Adams 1995).

Planteøstrogen har ein liknande struktur som endogent østrogen, østradiol (Figur 10). Planteøstrogen og østradiol har begge to hydroksyl grupper plassert med same avstand, samt ein fenylring som gjer det mogleg å binda seg til østrogenreseptorar i livmor, eggstokkar, hypothalamus og hypofyse (Adams 1995; Seested et al. 2000). Bindingsaffiniteten, og dermed graden av østrogeneffekt dei ulike planteøstrogena gjev, variera mellom planteøstrogena då strukturen er noko ulik (Cassidy et al. 2000). For coumestanar er bindingsaffiniteten til coumestrol om lag 1/20 av østradiol og for isoflavonar er den om lag 1/200 (Magee & Rowland 2004; Seested et al. 2000).

Planteøstrogen, og stoff danna med utgangspunkt i planteøstrogen, kan ha både østrogen og anti-østrogen effekt på metabolismen. Kva effekt som vert oppnådd er avhengig av konsentrasjonen av planteøstrogen, konsentrasjonen av endogent østrogen, kjønn og menopause status. Antiøstrogeneffekten kan bli forklart ved konkurranse mellom østradiol og

planteøstrogen om østrogenreseptorar (Tham et al. 1998). Det er forventa at ein i hovudsak ser østrogeneffekt hjå sauer, og antiøstrogeneffekt hjå menneskjer. Menneskjer har forholdsvis store konsentrasjonar av østrogen sirkulerande i kroppen, mens drøvtyggjarar har forholdsvis låge konsentrasjonar (Adams 1995). Kompleksa planteøstrogen dannar med østrogenreseptorar i cellene er ikkje like stabile som kompleksa danna med østrogen, difor er ikkje østrogeneffekta like sterk for planteøstrogen som for endogent østrogen (Seested et al. 2000). Østrogeneffekta for coumestanar er om lag 1/1000, og for isoflavonar er den om lag 1/10 000, av effekten av østradiol *(*Adams 1995*).*

2.4.1 Effekt på dyr og menneskjer

Østrogeneffekt av kløver vart først kjend i Australia tidleg på 1940 talet, der Bennetts et al.(1946) rapporterte at sauer som beita raudkløver fekk fertilitetsproblem (Cornwell et al. 2004; Cox & Braden 1974). Det er vist at stor tilførsel av planteøstrogen vil gje "clover disease" hjå sau. På førstegongslemmande søyer kan dette visa seg som vaginal prolaps eller bør-framfall. Drektighetsprosenten i besetninga blir låg, ein finn cystar i livmor og eggstokkar og embryodød dei første 30-60 dagar i drektigheita. For drektige søyer ser ein daudfødslar som følgje av dårleg opning på livmorhalsen og svake rier (Adams 1998; Cox & Braden 1974; Taponen et al. 2010).

Det er vist midlertidig nedsett fertilitet eller infertilitet på sau som følgje av redusert ovulering, noko som kan visa seg i få tvillingar og mange tomme søyer. Fertiliteten forbetrar seg 6-8 veker etter at sauene er tatt av diett med høgt innhald av planteøstrogen (Adams 1998). Ved langvarig påverknad av planteøstrogen kan det oppstå permanent infertilitet, utan symptom på "clover disease" (Taponen et al. 2010). Søyer som har eit kontinuerlig inntak av planteøstrogen over 4-5 månadar kan bli permanent infertile (Seested et al. 2000). Slike søyer ovulerar og blir brunstig som normalt men ein ser endringar i membranar på livmorhalsen samt at normal respons på endogent østrogen blir redusert, noko som fører til redusert viskositet i slim frå livmorhals og transport av spermiar. Inntak av planteøstrogen ser ikkje ut til og påverka fruktbarheten på værar, men på kastrerte værar kan jur kjertlar utvikla seg og mjølkeproduksjonen ta til (Adams 1998; Taponen et al. 2010).

Det er få kjende studiar på reproduksjonsproblem på storfe knytt til planteøstrogen. Omsetjing av planteøstrogen hjå storfe er liknande som for sau, men storfe ser ikkje ut til å bli påverka i like stor grad. Årsaka til dette er ukjend, men det er foreslått at storfe har større evne til å

omsetja planteøstrogen, og at storfe har færre østrogenreseptorar i livmor enn sau har (Taponen et al. 2010). Effektar av planteøstrogen på storfe er likevel rapporter. Både raudkløver surfôr og luserne har gitt infertilitet (Adams 1995). Feltforsøk har vist fertilitetsproblem på kyr, mjølkeproduksjon har teke til på kviger som har beita kløverrikt gras, og fôring med luserne har indusert utvikling av jur-vev og vekst i livmor. Data frå Finland viser at kyr fôra med raudkløver-surfôr hadde endringar i brunst syklus og stille eller manglande brunstar. Eit *In vitro* studie viser at equol og *para*-ethyl-phenol kan stimulera danninga av prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) i det gule legemet. $PGF_{2\alpha}$ forårsakar luteolyse i det gule legemet og dermed redusert progesteronproduksjon som er naudsynt for å oppretthalda drektighet (Taponen et al. 2010).

Kyr i negativ energibalanse kan vera ekstra sensitive for planteøstrogen, då nedbrytingsevna hjå vom mikrobane og leverfunksjonen kan vera nedsett, noko som aukar mengde ikkje nedbrotne og ikkje konjugerte planteøstrogen i blodet (Seested et al. 2000).

Effekt av formononetin på lam beitande på kløver er undersøkt, og viser at lam med eit høgt inntak (4,7 g/kg TS) av formononetin hadde ein større tilvekst (40 g/dag) enn lam med eit lågt inntak (0,0-3,3 g/kg TS). Årsaka til dette er at østrogen kan auka konsentrasjonen av vekst hormon og insuline-like growth factor-1 (IGF-1) (Moorby et al. 2004).

Inntak av planteøstrogen ser ut til å ha effekt på ei rekkje sjukdommar hjå menneskjer, t.d. ulike former for kreft, hjarte og kar sjukdommar og osteoporose. I hovudsak er det isoflavonar som er undersøkt, men det er også gjort nokre få studiar på lignanar (Cassidy et al. 2000). Mange av dei potensielt positive helseeffektane kan skuldast metabolske eigenskapar som ikkje er avhengige av østrogenreseptorar, slik som t.d. enzym, protein syntese, cellevekst, utvikling av nye blodårer, kalsium transport og celledeling (Tham et al. 1998).

Brystkreft er den vanlegaste kreftforma blant norske kvinner (Cancer Registry of Norway 2009). Fullstendig årsak til brystkreft er ikkje klarlagt, men aukande mengde østrogen i serum og påverknad av østrogen etter overgangsalder ser ut til å gje auka risiko (Clemons & Goss 2001; Thomas et al. 1997). I eit studium av japanske kvinner ser det ut til at inntak av planteøstrogen kan redusera faren for brystkreft (Adlercreutz 2003). Eit større inntak av planteøstrogen og lange menstruasjonssyklusar gjev ein mindre påverknad av østrogen gjennom livet, og kan dermed gje ein lågare fare for å få brystkreft (Cassidy et al. 2000). Eit høgare inntak av Vitamin D hjå forsøkspersonane kan også redusera faren for brystkreft, så årsakssamanhengen her er truleg samansett (Adlercreutz 2003).

In vitro studiar med kreftceller frå tjukktarmen har vist at enterodiol og enterolactone kan hemme vekst av kreftceller, mens østradiol ikkje har nokon effekt (Cassidy et al. 2000). Det ser heller ikkje ut til at isoflavonoidar har noko førebyggjande effekt på denne krefttypen (Adlercreutz 2002).

Det er vist at menn med høgt inntak av isoflavonar, er høge konsentrasjonar av isoflavonar i plasma kan ha lågare risiko for prostatakreft (Cassidy et al. 2000).

Hjarte og karsjukdommar ser ut til å vera eit problem for kvinner hovudsakeleg etter overgangsalder, og det ser ut til at endogen østrogen kan ha ein vernande effekt (Cassidy et al. 2000). Eit høgt inntak av planteøstrogen og lite hjarte/karsjukdommar hjå den asiatiske befolkninga kan bety at planteøstrogen har ein fordelaktiv effekt på hjarte/karsjukdommar. Samtidig har det asiatiske kosthaldet eit innhald som også verkar positivt, eks lite metta feitt, så det er vanskeleg å trekkja konklusjonar (Tham et al. 1998).

Osteoporose er ein sjukdom knytt til redusert beintettleik, og er for kvinner i hovudsak knytt til overgangsalder då manglande østrogenproduksjon aukar tap av beinmasse. Studiar viser at inntak av isoflavonar over tid aukar beintettleik og beinmineralinnhaldet hjå kvinner i starten av overgangsalderen (Cornwell et al. 2004).

Planteøstrogen blir også knytt til negative helseeffektar, særskild spedbarnsutvikling. Steroidar påverkar modning av skjelett og vekst, men studiar har ikkje funne ein klar samanheng mellom inntak av planteøstrogen og normal vekst på friske spedbarn. På mus er det vist at ved påverknad av isoflavonar i fosterliv og under dieperioden gav reduksjon i storleik på eggstokkar og livmor på avkomma (Zung et al. 2001).

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3.0 Metabolism of phytoestrogens in dairy cows – effect of botanical composition of silage.

3.1 Introduction

Phytoestrogens is a large group of plant-derived non-steroidal compounds, with structural similarities to mammalian estrogen (17-β Estradiol). Phytoestrogens can bind to estrogen receptors and provide a weak estrogenic and/or anti-estrogenic effect (Sirtori et al. 2005; Tham et al. 1998). Effect of phytoestrogens was first shown in the 1940s, where Bennetts et al. (1946) reported that sheep grazing on pastures containing red clover had fertility problems (Cornwell et al. 2004; Cox & Braden 1974). The interest in phytoestrogen is now linked to their possible favorable influence on health disorders, such as cardiovascular disease, hormone-related cancers, osteoporosis and menopausal symptoms, but also possible adverse health effects on infant development and processes influenced by sex steroids (Cornwell et al. 2004; Mendez et al. 2002; Tham et al. 1998; Tuohy 2003; Zung et al. 2001).

Most studies are conducted on vegetables as a source of phytoestrogen, and there are only a few studies on products of animal origin. Ruminant feedstuff may contain phytoestrogens, mainly isoflavones, coumestans and lignans, which can be transferred to milk after digestion (Andersen et al. 2009). Soy in concentrate supplements and grassland legumes, such as red clover, are the most important sources of isoflavones in ruminant feedstuff. Lignans are primarily found in cereals, legumes and seeds used in concentrate, and are a constituent of the cell wall (Saloniemi et al. 1995; Tham et al. 1998). Coumestrol are the most studied coumestan, mainly found in clover and lucerne (Magee & Rowland 2004).

Phytoestrogens are mainly present in plant material as a glycoside, which is not active estrogenically (Bingham et al. 1998). The major metabolic transformation of isoflavones is performed by rumen microbes, which hydrolyses glycosides. Biochanin A is demethylated to genistein, which both provide estrogenic effect themselves, are further metabolized via ring cleavage to p-ethyl phenol and organic acids without estrogenic effect. Formononetin is mainly demethylated to daidzein and further to equol via hydrogenation and ring fission. Formononetin itself provide little or no estrogenic effect, while the metabolite equol can give estrogen effect (Cox & Braden 1974; Lundh 1995). Coumestrol does not seem to be metabolized in rumen, but provide estrogenic effect itself. Isoflavones are mainly absorbed in rumen, and conjugated with glucuronic acid in rumen gastro intestinal epithelium (Seested et

al. 2000). Metabolism of lignans is similar to the metabolism of isoflavones. The major lignans found in plant material is matairesinol and secoisolariciresinol (Magee & Rowland 2004). Rumen microbes convert glycosides, as secoisolariciresinol-diglucoside, to aclycones which is further converted to mammalian lignans, enterodiol and enterolactone. Absorption occur either in rumen or in the small intestine before enterodiol could be further converted to enterolactone by colon microbes (Côrtes et al. 2008; Gagnon et al. 2009a). Mammalian lignans are reconjugated with sulfate or glucuronic acid in the intestine wall or liver, excreted in plasma, urine or the bile duct. The conjugated mammalian lignans are poorly absorbed in the intestine, however being imminent cleaved by microbial enzymes such as β-glucuronidase and reabsorbed (Gagnon et al. 2009a; Tham et al. 1998).

Several have shown that content of phytoestrogens in milk can be affected by content in feed. Kalăc (2011) have summarized studies regarding phytoestrogen content in milk and the total content of phytoestrogens ranged from 56-412 µg/l, however content of equol has be reported as high as 643 µg/l (Mustonen et al. 2009). Highest content of phytoestrogens was found in milk from cows fed red-clover grass silage. Equol was the dominating in milk from diets from red clover, while lignans was dominating in milk from white clover or lucerne pasture (Kalăc 2011). Steinshamn et al. (2008) and Andersen et al. (2009) showed that concentration of phytoestrogens in milk can be modulated by concentration of phytoestrogens and their precursors in feed.

The main objective of this study was to investigate the metabolism and metabolic pathway of phytoestrogens in dairy cows fed different diets, and the possibility to influence the content of phytoestrogens in milk.

3.2 Material and methods

3.2.1 Experimental design and animals

The experiment was carried out at the Department of Animal and Aquacultural sciences, Norwegian University of Life Science with four fistulated dairy cows. At the start of the experiment the cows weighed on average 631 kg (SD ± 35.5 kg), were 118 days into lactation (SD ± 40.9 days) and yielded 24 kg milk daily (SD ± 2.4 kg). The cows where randomly allocated in a 4 x 4 latin square experimental design, one cow per treatment and period. Each experimental period lasted for 21 days, 14 days adaptation and 7 days sample collection. They were housed in a tied stall, and where milked twice a day, at 6.30 and 15.30.

3.2.2 Feeds and feeding

Four silages with different botanical composition where used; grass-red-clover, botanical diverse, perennial ryegrass and timothy. Botanical composition was manually recorded by sorting, prior to each harvest, by harvesting 6 squares, each of 0.5 m^2, per meadow (Table 1). First cut was taken at early heading stage of timothy, then second and third with 6-7 weeks intervals. A mixture of first and third harvesting was used as treatment diets. Second cut was not possible to use, because of drought and low yields.

Table 1. Botanical composition in percentage of total dry matter (DM) yield of the experimental silages

	Latin name	Red clover	Botanical diverse	Perennial ryegrass	Timothy
DM concentration after wilting, g DM kg^{-1}		328	278	301	340
Timothy	*Phleum pratense*	41.96	17.64	0.00	95.69
Perennial ryegrass	*Lolium perenne*	1.22	1.21	87.30	0.00
Meadow fescue	*Festuca pratensis*	0,00	10,75	0,00	0,00
Smooth Meadow-grass	*Poa pratensis*	0.32	25.66	0.00	0.00
Other grasses		8.61	7.30	0.25	0.30
Red Clover	*Trifolium pretense*	36.28	1.45	0.21	0.00
White Clover	*Trifolium repens*	2.65	8.99	5.99	0.11
Alsike Clover	*Trifolium hybridum*	0.07	0.00	0.00	0.00
Tufted vetch	*Vicia cracca*	0.05	0.00	0.00	0.08
Dandelion	*Taraxacum officinale*	0.38	9.49	0.00	0.00
Creeping buttercup	*Ranunculus repens*	0.60	9.02	0.00	0.00
Other herbs		1.97	4.83	0.01	0.00
Dead material		5.88	3.65	6.24	3.81

The used concentrate contained organically produced barley (93.3 %), organically produced molasses (5 %) and minerals with Selenium (1.7 %, Vilomix, Norsk Mineralnæring). Concentrate and silage were feed at constant ratio 30:70 on dry matter (DM) basis through the experiment. Shortening indicates diet; grass-red-clover (RC), botanical diverse (BD), perennial ryegrass (PR) and timothy (TI).

The cows were fed individually with automatically belt feeders 4 times per day, with 6 hours between each feeding. Concentrate and silage were fed together on same time. Feed were given *ad libitum* from day 1-11, and 90 % of *ad libitum* intake from day 12-21. Feed intake was recorded every day, and feed leftovers were collected from day 12-21. Rumen content was exchanged between cows with same treatment on day one in subsequent period to facilitate adaption to new silage.

3.2.3 Triple marker system

Marker technique was used to determine digesta flow to the omasal canal. Indigestible neutral detergent fiber (iNDF) was used as internal marker for large particles, Ytterbium acetate (YbAc) and Chromium ethylene diamine tetra-acetic acid (CrEDTA) was used as external markers for small particles and liquid, respectively. YbAc (2.98 g/d Yb) and CrEDTA (3.04 g/d Cr) was dissolved in tap water and infused directly into the rumen of each cow from day 8-20, using a peristaltic pump (Cenco instruments MIJ. N.V., Breda, the Netherlands) with tubes to the rumen. Before infusion start, a dose equal to half the daily amount of both external markers was given to each cow, to quickly reach equilibrium of marker in rumen.

3.2.4 Sampling and analyses

Feeds

Samples from each silage type and feed residues were collected daily from day 15 to day 20 and stored frozen. At the end of each period, the five samples were mixed, pooled to one sample per silage and prepared for analysis. Freeze dried sample (Christ LCM-2 beta 1-16 and Christ LOC-1 m alfa 1-4, Martin Christ, Osterode am Harz, Germany; Hetosicc, Birkerød, Denmark) were ground on a hammer mill (<1 mm) and analyzed for DM, Ash, Kjeldahl-Nitrogen (Kjeldahl-N), neutral detergent fibre (NDF), phytoestrogens, starch (<0.5 mm) and iNDF (<1.5 mm). Raw sample were analyzed for DM, Kjeldahl-N, ammonia-nitrogen (NH_3-N), pH, volatile acids and ethanol. Loss of volatile compounds in silage during drying was corrected for by equations from the Nordic Feed Evaluation System (Åkerlind et al. 2011).

Three samples of concentrate was collected in each period, pooled, stored frozen and analyzed for DM, Ash, Kjeldahl-N, NDF, starch and phytoestrogens.

Markers

Samples of infusing fluid was collected at day 12, 17 and 21, and stored at 4 °C before analyses.

Omasum digesta

To determine digesta flow to the omasal canal, samples of omasal canal digesta were collected according to the technique developed by Huhtanen (1997) and modified by Ahvenjärvi (2000). 500 ml digesta was collected when leaving the reticulo-rumen, and led to outside the rumen cannula through a plastic tube using a vacuum pump. 12 samples were taken from each cow on day 18-20, 4 per day distributed so that there is one sample every two hours for 24 hours. Samples were pooled and stored frozen until preparation. Preparation was done according to method described by Krizsan (2010) where digesta was divided into three different phases; large particles (LP), small particles (SP) and fluid phase (FP), by filtering through a 100 µm nylon filter and centrifuged at 1,000 × g for 10 min at 5°C. After preparation the different phases was frozen until freeze drying, and milled. LP and SP (<1.0 mm) were analyzed for iNDF, Yb, Cr, DM, and phytoestrogens, FP were analyzed for DM, Yb, Cr, and phytoestrogens.

Feces and urine

Feces were collected quantitatively from each cow 3 times per day in 3 days, and stored chilled between collections. 10 % of daily fecal production was pooled to one sample per collection period and stored frozen. DM was analyzed in raw material, DM, phytoestrogens, Yb, Cr (milled, <1.0 mm) and iNDF (milled, <1.5 mm) were analyzed in freeze dried material.

The cows were fitted with urine funnels that led to collection buckets. Urine was collected quantitatively from each cow 3 times per day in 3 days. 0.5 l 10% H_2SO_4 were added to collection buckets after emptying to ensure a pH below 4.0. 10 % of daily urine production was pooled to one sample per collection period. Urine was analyzed for Cr and phytoestrogens.

Milk

Milk yield was weighed at each milking. Milk samples was taken 3 days in the sample collection period, pooled and stored at 0-2 °C. Pooled samples were stored frozen until analysis for the content of phytoestrogens.

3.2.5 Analysis

Chemical analysis of feed

DM was analyzed by drying at 105 ºC until constant weight, 4 hours or overnight. Ash was analyzed by flame combustion by heating to 550 ºC h hours or overnight. Kjeldahl-N was analyzed after method described in AOAC, number 984.13 (1990), with following modification: digestion with 15 ml H_2SO_4, 3.5 g K_2 SO_4, and 0.4 g Cu SO_4, boiled in 45 min at 420 ºC on heating block and added 250 ml distilled water after cooling. Content of NDF was determined after Mertens (2002). Content of starch was determined by method described in AACC (2000) with following modifications: the enzyme pullulanase was not used, heat stable α-amylase was used. iNDF was determined by 288 hour *in sacco* incubation in nylon bags (17 µm) in two cows. After incubation the bags where washed in cold water in a laundry machine. Volatile compounds were detected by infrared detector, conducted by Eurofins dep. Moss.

Chemical analysis of markers

Yb and Cr in marker infusing fluid was determined after method described by Udén et al. (1980) with following modification: 50 µl H_2SO_4 was added. For analysis of Yb and Cr in omasum and feces same method was used (Udén et al. 1980), with following modifications for Yb: 200 mg sample material was dissolved in scintillation-glasses, boiled for 30 minutes and diluted to 21.5 ml. and for Cr: 100 mg sample material was combusted and dissolved with 0.75 ml H_3PO_4 and 1 ml $KBrO_3$ in scintillation-glasses, boiled for 10 minutes and diluted to 21.5 ml.

Chemical analysis of phytoestrogen

Analysis of phytoestrogens was done by the Department of Animal Health, Welfare and Nutrition, Faculty of Agricultural Sciences, University of Aarhus according to the method

described by Steinshamn et al. (2008). Feed (silage, feed residues and concentrate), omasum and feces- samples were extracted with ethanol and acetate buffer (pH 5.0), then incubated with Cellulase Onozuka R-10 from *Trichoderma viride* (Merck, Darmstadt, Germany) in ambient temperature overnight.

Urine was centrifuged at 3000 *x g* for 10 min, and then a sample (500 µl) was deprotenized by mixing with 500 µl acetate buffer (pH 5.2). Conjugates of phytoestrogens were cleaved by incubation with 5 µl β-glucuronidase and sulfatase from *Helix pomatia* type H2 (Sigma/Aldrich, St. Louis) at 37 °C for 1 hour.

Milk was deprotenized and defatted using acetate buffer (pH 5.2), heptane and acetone. Acetone/water phase was evaporated to dryness and residues re-dissolved in water. Conjugates of phytoestrogens were cleaved by incubation with β-glucuronidase and sulfatase from *Helix pomatia* type H2 (Sigma/Aldrich, St. Louis) at 40 °C for 4 hours.

Secoisolariciresinol, mateiresinol, enterodiol, daidzein, enterolacton, equol, genistein, coumestrol, formononetin, prunetin and biochanin A were analyzed using the liquid chromatography (LC)-mass spectrometry (MS)/MS technique, where feed, omasum and feces were analyzed with extern standard. Urine and milk were analyzed with standard addition. This method for analysis of the content of phytoestrogen in urine is insecure because the method has not been validated for analysis of urine.

Statistical analysis

Data were analyzed using the GLM procedure in Minitab 15(MINITAB 2007) by the following model: $Y_{ijk} = \mu + \alpha_i + \beta_j + c_k + e_{ijk}$

where *Y* is the dependent variable, μ the mean, α the fixed effect of period *i* (1-4), β the fixed effect of treatment *j* (1-4), *c* the random effect of cow *k* (1-4). Analyses of phytoestrogen concentration in silages were done without *k* in the model. For the analysis of the flow of phytoestrogens to omasum, phase (1-3) and its interaction with diet were included as fixed effects. Tukey's multiple comparison test was used to determine significant difference between treatments.

If the content of phytoestrogens was below detection limit, half the detection limit was used to be able to run the statistical analysis.

3.2.6 Calculations

Feed intake was calculated as difference between DM offered and DM residue. Crude protein was calculated as Kjeldahl-N x 6.25. Digesta flow to omasum was calculated using the tree marker system according to the reconstitution technique by Faichney (1975) and Armentano and Russell (1985). Amount concentrate in residue was estimated using starch content in residue. The amount of phytoestrogens with similar metabolic pathway were merged when calculating the total metabolism; Biochanin A and genistein was merged in BG, formononetin, daidzein and the metabolite equol merged in FDE and the lignans secoisolariciresinol, matairesinol, enterodiol and enterolactone was merged in SMEE.

3.3 Results

3.3.1 Feed composition

Content of some chemical compounds of the four silages and concentrate is shown in Table 2.

Table 2. Chemical composition of silages, concentrate, pH and volatile compounds in silages

	Silage								Concentrate	
	Red clover		Botanical divers		Perennial ryegrass		Timothy			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N	4		4		4		4		4	
DM, g kg^{-1}	328	5.7	271	5.4	290	8.1	322	18.9	901	2.1
Chemical composition, g kg^{-1} DM										
Ash	80	2.5	97	26.4	85	2.3	69	2.3	45	4.0
NDF	394	7.4	400	9.5	380	11.7	493	12.7	168	3.5
Crude protein	138	8.18	122	6.61	147	5.86	106	7.09	125	1.25
Starch	32	3.4	17	1.4	20	2.6	30	24.2	510	11.3
Volatile compounds, g kg^{-1} DM										
Lactic acid	31.0	2.04	62.9	1.25	60.8	2.06	34.4	10.7		
Acetic acid	6.3	0.56	10.0	0.39	9.9	0.49	6.4	0.47		
Butyric acid	0.8	0.28	0.9	0.21	0.6	0.08	0.5	0.34		
Propionic acid	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00		
Ammonium-N	1.3	0.10	1.6	0.06	2.0	0.21	1.6	0.11		
Ethanol	14.8	2.13	18.6	2.69	19.7	2.64	16.1	2.12		
pH	4.5	0.00	4.4	0.06	4.5	0.05	4.7	0.17		

DM=dry matter; NDF= neutral detergent fiber.

SD= standard deviation

There was no significant difference in DM content between silages, but timothy distinguished from the others with a higher content of NDF and a lower content of crude protein. Content of starch was lower in botanical diverse and perennial ryegrass silage than in red clover and

timothy silage. Fermentation quality measured by the most common volatile compounds showed a higher content of lactic acid in the silages with low content of starch, beyond this there was small differences.

3.3.2 Feed intake, omasum flow and feces, urine and milk output

Feed intake, omasum, exertion of feces and urine and milk production are shown in Table 3.

Table 3. Feed intake, omasum flow of large particles (LP), small particles (SP), fluid phase (FP) and total omasum flow, fecal and urine output and milk yield for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI), where N = 4 for all treatments

	Treatment					
	RC	BD	PR	TI	SED	p-value
Feed intake, kg DM/day						
Silage	12.5	12.5	12.2	11.6	0.522	0.352
Concentrate	5.42	5.42	5.21	5.10	0.180	0.286
Total	17.9	17.9	17.4	16.7	0.654	0.295
Omasum flow, kg DM/day						
LP	4.23	4.44	3.95	5.27	0.783	0.435
SP	1.58	1.00	1.01	0.90	0.225	0.075
FP	4.20	3.90	4.09	4.79	0.635	0.571
Total	10.0	9.34	9.05	11.0	1.38	0.562
Output						
Feces kg, DM/day	3.28	3.44	3.09	3.31	0.252	0.598
Urine, l/day	16.7	20.5	20.8	17.0	1.37	0.043
Milk, kg/day	17.8[a]	18.4[a]	17.9[a]	16.3[b]	0.259	0.001

[a,b] Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Intake of silage was similar in RC, BD and PR, but lower in TI. This was reflected in a significant lower milk yield in TI compared to the other diets. There was a tendency (*p=0.075*) that the flow of small particles to duodenum was higher on RC than other diets. Diet affected the excretion of urine, with the lowest values in RC and TI.

3.3.3 Concentration of phytoestrogens in feed, digesta, feces, urine and milk

Concentrations of phytoestrogens in silages are shown in Table .

Table 4. Concentration of phytoestrogens in concentrate and experimental silages; red-clover, botanical diverse, perennial ryegrass and timothy. N = 4 for all silages

	Silages						Concentrate
	Red clover	Botanical diverse	Perennial ryegrass	Timothy	SED	p-value	
Feed mg/kg DM							
Isoflavones							
Biochanin A	1 306[a]	51.2[b]	2.63[b]	< 0.20[b]	17.5	< 0.001	< 0.19
Genistein	210[a]	8.09[b]	2.71[b]	< 0.123[b]	7.79	< 0.001	7.57
Formononetin	3 015[a]	211[b]	40.4[c]	7.81[c]	34.4	< 0.001	< 0.07
Daidzein	63.0[a]	2.43[b]	0.54[b]	0.13[b]	2.80	< 0.001	4.97
Prunetin	300[a]	8.80[b]	0.305[b]	< 0.06[b]	11.9	< 0.001	< 0.06
Lignans							
Matairesinol	2.58[b]	1.91[b]	< 0.35[c]	7.90[a]	0.271	< 0.001	< 0.32
Secoisolariciresinol	4.43[b]	6.40[b]	11.5[a]	9.88[a]	0.699	< 0.001	1.02
Coumestans							
Coumestrol	1.03[a]	1.48[a]	< 0.37[b]	< 0.49[b]	0.162	< 0.001	< 0.31

[a,b,c] Different letters within row indicates statistical difference between treatment (*p<0.05).* SED= standard error deviation.

Concentration of isoflavones was higher than lignans and coumestrol in red-clover, botanical diverse and perennial ryegrass silage, whereas for timothy the concentration of lignans was higher than isoflavones and coumestrol. Red clover silage had higher concentration of all isoflavones than the other silages. The content of the lignan mateiresinol was significantly higher in timothy silage than in the other silages, while the pure grass silages (timothy and perennial ryegrass) had higher content of secoisolariciresinol than the silages with clover (Table 1). The content of coumestrol was higher in red clover and botanical diverse silage, than in the grass based silages.

Concentration of phytoestrogens in omasum and feces are shown in Table 5.

Table 5. Concentration of phytoestrogens in omasum and feces for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI), where N = 4 for all treatments

	Treatment					
	RC	BD	PR	TI	SED	p-value
Omasum mg/kg DM						
Isoflavones						
Biochanin A	11.2[a]	0.205[b]	0.212[b]	0.203[b]	2.13	0.005
Genistein	1.34[a]	0.126[b]	0.130[b]	0.125[b]	0.184	0.001
Formononetin	68.7[a]	2.32[b]	0.585[b]	0.041[b]	10.5	0.001
Daidzein	8.36[a]	0.466[b]	0.298[b]	0.207[b]	1.64	0.006
Equol	504[a]	17.9[b]	4.25[b]	1.45[b]	22.5	< 0.001
Prunetin	2.53[a]	0.062[b]	0.064[b]	0.062[b]	0.275	< 0.001
Lignans						
Matairesinol	0.407	0.350	0.326	0.320	0.036	0.158
Secoisolariciresinol	0.664	0.659	0.670	0.664	0.012	0.848
Enterolactone	96.1	92.7	109	127	14.2	0.168
Enterodiol	0.933	0.823	0.843	0.879	0.2	0.947
Coumestans						
Coumestrol	0.787[a]	0.795[a]	0.441[b]	0.338[b]	0.055	< 0.001
Feces mg/kg DM						
Isoflavones						
Biochanin A	2.08	<0.198	< 0.199	< 0.199	0.777	0.121
Genistein	0.45	<0.122	< 0.122	< 0.122	0.235	0.465
Formononetin	20.8[a]	0.783[b]	< 0.077[b]	< 0.077[b]	2.67	0.001
Daidzein	4.24[a]	0.488[b]	0.366[b]	0.267[b]	0.885	0.011
Equol	349[a]	11.9[b]	4.19[b]	1.35[b]	38.4	< 0.001
Prunetin	0.850[a]	<0.060[b]	< 0.060[b]	< 0.060[b]	0.19	0.014
Lignans						
Matairesinol	< 0.33	<0.34	< 0.34	<0.34	0.01	0.323
Secoisolariciresinol	< 0.62	<0.64	< 0.64	<0.64	0.01	0.323
Enterolactone	164	160	226	251	30	0.052
Enterodiol	2.06[b]	2.17[b]	3.72[a]	3.04[ab]	0.30	0.004
Coumestans						
Coumestrol	0.60[b]	0.786[a]	0.357[c]	< 0.330[c]	0.04	< 0.001

[a,b,c] Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Effect of diet was shown for isoflavones and coumestrol in the digesta, where red clover silage had higher concentration of all isoflavones. In feces the effect of diet on the concentration of biochanin A and genistein had diminished, but for formononetin, equol, daidzein and prunetin the effect of RC was still present yielding higher concentration than

other diets. Equol was the dominating isoflavones and enterolactone the dominating lignan in feces from all diets. The concentration of enterodiol in feces was higher in cows fed the grass silages than in feces from RC and BD.

Concentration of phytoestrogens in urine and milk are shown in Table 6.

Table 6. Concentration of phytoestrogens in urine and milk for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI), where N = 4 for all treatments

	Treatment					
	RC	BD	PR	TI	SED	p-value
Urine µg/l						
Isoflavones						
Biochanin A	16.0	7.02	4.20	169.6	53.5	0.056
Genistein	10.0	8.71	5.94	17.7	3.88	0.097
Formononetin	55.6^{ab}	40.0^{ab}	11.7^{b}	806^{a}	224	0.032
Daidzein	115	65.6	28.7	126	42.3	0.179
Equol	27 273^{a}	5 217^{b}	246^{b}	52.1^{b}	3 971	0.001
Prunetin	3.65	1.51	0.830	13.6	4.22	0.073
Lignans						
Matairesinol	21.3	1.87	4.89	2.95	11.3	0.355
Secoisolariciresinol	0.787	0.979	0.685	0.480	0.321	0.520
Enterolactone	231^{b}	5 358^{a}	5 942^{a}	5 954^{a}	527	< 0.001
Enterodiol	14.5^{d}	65.0^{b}	80.1^{a}	52.6^{c}	2.31	< 0.001
Coumestans						
Coumestrol	9.98	11.5	3.21	5.95	3.30	0.145
Milk µg/l						
Isoflavones						
Biochanin A	1.69^{a}	0.345^{b}	0.166^{b}	0.120^{b}	0.207	0.001
Genistein	3.31^{a}	2.44^{ab}	2.50^{ab}	1.68^{b}	0.307	0.011
Formononetin	8.20^{a}	2.80^{b}	2.33^{b}	1.63^{b}	0.739	< 0.001
Daidzein	4.69^{a}	1.67^{b}	1.28^{b}	0.948^{b}	0.328	< 0.001
Equol	442^{a}	50.1^{b}	12.8^{b}	3.90^{b}	40.7	< 0.001
Prunetin	1.01	0.729	0.671	0.345	0.204	0.086
Lignans						
Matairesinol	0.947	1.13	0.910	1.16	0.342	0.843
Secoisolariciresinol	3.66	4.25	3.24	5.30	1.15	0.383
Enterolactone	17.9^{b}	39.3^{ab}	23.3^{ab}	46.7^{a}	7.15	0.021
Enterodiol	0.468	0.408	0.471	0.484	0.158	0.963
Coumestans						
Coumestrol	< 0.102	< 0.102	< 0.102	< 0.102		

^{a,b,c,d} Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Effect of diet was shown for the concentration of formononetin and equol in urine, and for all isoflavones, except prunetin, in milk. Equol was the dominating isoflavone in urine from RC, BD and TI, while formononetin was the dominating for PR. The concentration of equol was highest in urine from cows fed RC (27 273 µg/liter). In milk, the highest concentration for all isoflavones, except prunetin, was found in cows fed RC (total: 461µg/l), whereas TI gave the lowest (total: 8.63 µg/l). RC and BD gave a higher concentration of isoflavones than lignans in milk, while PR and TI gave a higher concentration of lignans than isoflavones.

The mammalian lignan enterodiol was significantly higher in urine for PR than from other diets. Enterolactone concentration was lower in urine from cows fed RC than urine from the other diets. In milk there was significant difference in concentration of enterolacton, where TI had highest concentration and RC the lowest. For the other lignans there was no difference in concentration between diets.

The urine content of coumestrol was not affected by diet and coumestrol was not detectable in milk.

3.3.4 Intake, flow and excretion of phytoestrogens

Intake of phytoestrogens, amount passing to omasum and content in feces is shown in Table 7.

Table 7. Intake, passage of phytoestrogens in omasum and excretion in feces per day, for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI), where N = 4 for all treatments

	Treatment					
	RC	BD	PR	TI	SED	p-value
Intake mg/day						
Isoflavones						
Biochanin A	16 251[a]	649[b]	35.3[b]	3.30[b]	816	< 0.001
Genistein	2 653[a]	143[b]	73.7[b]	39.6[b]	156	< 0.001
Formononetin	37 465[a]	2 651[b]	498[b]	94.2[b]	1 712	< 0.001
Daidzein	818[a]	57.4[b]	33.0[b]	26.7[b]	64.1	< 0.001
Prunetin	3 710[a]	111[b]	4.43[b]	1.00[b]	146	< 0.001
Lignans						
Matairesinol	34.5[b]	25.92[b]	5.89[c]	93.0[a]	4.87	< 0.001
Secoisolariciresinol	62.2[b]	85.2[b]	144[a]	120[a]	7.74	< 0.001
Coumestans						
Coumestrol	14.8[ab]	20.4[a]	6.21[b]	7.02[b]	2.64	< 0.001
Omasum mg/day						
Isoflavones						
Biochanin A	102[a]	1.92[b]	1.92[b]	2.23[b]	11.2	< 0.001
Genistein	12.7[a]	1.18[b]	1.18[b]	1.37[b]	0.987	< 0.001
Formononetin	642[a]	21.8[b]	5.18[b]	0.448[b]	41.9	< 0.001
Daidzein	76.9[a]	4.71[b]	2.67[b]	2.18[b]	7.93	< 0.001
Equol	4 991[a]	168[b]	38.1[b]	16.3[b]	331	< 0.001
Prunetin	24.1[a]	0.581[b]	0.582[b]	0.674[b]	0.916	< 0.001
Lignans						
Matairesinol	3.94	3.31	2.99	3.53	0.675	0.589
Secoisolariciresinol	6.65	6.17	6.08	7.30	0.958	0.595
Enterolactone	962	885	993	1 370	197	0.164
Enterodiol	9.16	7.90	7.68	9.17	2.56	0.896
Coumestans						
Coumestrol	7.89	7.41	4.12	3.70	1.30	0.036
Feces mg/day						
Isoflavones						
Biochanin A	7.41	0.683	0.618	0.658	2.90	0.138
Genistein	1.74	0.419	0.379	0.404	0.969	0.473
Formononetin	66.6[a]	2.67[b]	0.238[b]	0.254[b]	7.15	< 0.001
Daidzein	13.4[b]	1.70[b]	1.10[b]	0.883[b]	2.58	0.007
Equol	1 116[a]	39.9[b]	12.9[b]	4.38[b]	95.2	< 0.001
Prunetin	2.89[a]	0.207[b]	0.187[b]	0.199[b]	0.694	0.019
Lignans						
Matairesinol	1.08	1.16	1.05	1.11	0.097	0.708
Secoisolariciresinol	2.04	2.19	1.98	2.11	0.184	0.708
Enterolactone	557	550	697	837	122	0.154
Enterodiol	6.82 [b]	7.48 [b]	11.3[a]	10.1[b]	1.087	0.017
Coumestans						
Coumestrol	1.97[b]	2.69[a]	1.12[c]	1.09[c]	0.186	< 0.001

[a,b,c] Different letters within row indicates statistical difference between treatment (*$p<0.05$).*

SED= standard error deviation.

Excretion of phytoestrogens in milk and urine are shown in Table 8.

Table 8. Excretion of phytoestrogens in urine and milk per day, for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI), where N = 4 for all treatments

	Treatment					
	RC	BD	PR	TI	SED	p-value
Urine mg/day						
Isoflavones						
Biochanin A	0.274	0.145	0.086	2.96	1.05	0.087
Genistein	0.170	0.175	0.121	0.302	0.076	0.201
Formononetin	0.883	0.849	0.242	13.9	4.36	0.053
Daidzein	1.97	1.35	0.579	2.09	0.787	0.297
Equol	446[a]	103[b]	5.27[b]	0.892[b]	75.6	0.003
Prunetin	0.065	0.031	0.017	0.236	0.081	0.107
Lignans						
Matairesinol	0.387	0.038	0.103	0.051	0.210	0.382
Secoisolariciresinol	0.013	0.020	0.014	0.008	0.005	0.282
Enterolactone	4.29[b]	105[a]	119[a]	97.8[a]	7.80	< 0.001
Enterodiol	0.229[c]	1.28[ab]	1.60[a]	0.858[b]	0.126	< 0.001
Coumestans						
Coumestrol	0.156	0.230	0.063	0.100	0.052	0.073
Milk mg/day						
Isoflavones						
Biochanin A	0.029[a]	0.007[b]	0.003[b]	0.002[b]	0.004	0.001
Genistein	0.057[a]	0.044[ab]	0.043[ab]	0.027[b]	0.006	0.009
Formononetin	0.142[a]	0.050[b]	0.040[b]	0.026[b]	0.013	< 0.001
Daidzein	0.082[a]	0.030[b]	0.022[b]	0.015[b]	0.007	< 0.001
Equol	7.61[a]	0.884[b]	0.219[b]	0.060[b]	0.713	< 0.001
Prunetin	0.018	0.013	0.011	0.006	0.004	0.081
Lignans						
Matairesinol	0.017	0.020	0.016	0.018	0.006	0.865
Secoisolariciresinol	0.062	0.076	0.055	0.082	0.018	0.481
Enterolactone	0.317	0.731	0.403	0.739	0.137	0.044
Enterodiol	0.008	0.007	0.008	0.007	0.003	0.974
Coumestans						
Coumestrol	0.002[a]	0.002[a]	0.002[a]	0.002[b]	0.000	0.001

[a,b,c] Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Intake, flow to omasum and the amount excreted in feces, urine and milk of phytoestrogens per day (Table 7 & 8) reflects the concentration in Table 4, 5 and 6. Intake is a product of

concentration in feed and feed intake, and due to small differences in feed intake (Table 3), intake of phytoestrogens (Table 7) reflects the concentration in feed (Table 4). The same applies for flow to omasum, content in feces, urine and milk.

Coumestrol was not detectable in milk, and the difference in secretion through milk was due to different milk yield between treatments.

3.3.5 Metabolism of phytoestrogens in dairy cows

Omasal flow of phytoestrogens

The amount of phytoestrogens in different phases of omasum is shown in Table 9.

Table 9. Content of phytoestrogens in omasum phases, large particles (LP), small particles (SP) and fluid phase (FP) for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI), where N = 4 for all treatments

Phase	Treatment					p-value		
	RC	BD	PR	TI	SED	Phase	Diet	Phase*Diet
Biochanin A, mg/day								
LP	88.2[a]	< 0.88[b]	< 0.814[b]	< 1.07[b]	6.54	< 0.001	< 0.001	< 0.001
SP	13.1[b]	< 0.206[b]	< 0.205[b]	< 0.179[b]				
FP	< 0.907[b]	< 0.833[b]	< 0.903[b]	< 0.982[b]				
Genistein mg/day								
LP	10.5[a]	< 0.541[bc]	< 0.500[bc]	< 0.654[bc]	0.393	< 0.001	< 0.001	< 0.001
SP	1.65[b]	< 0.126[c]	< 0.126[c]	< 0.110[c]				
FP	0.557[bc]	< 0.512[bc]	< 0.554[bc]	< 0.603[bc]				
Formononetin mg/day								
LP	530[a]	14.2[c]	4.19[c]	< 0.000[c]	18.1	< 0.001	< 0.001	< 0.001
SP	78.8[b]	3.00[c]	0.637[c]	< 0.069[c]				
FP	33.4[bc]	4.60[c]	< 0.348[c]	< 0.379[c]				
Daidzein mg/day								
LP	22.6[b]	2.62[c]	1.62[c]	0.898[c]	3.94	< 0.001	< 0.001	< 0.001
SP	7.91[c]	0.396[c]	0.322[c]	0.112[c]				
FP	46.3[a]	1.69[c]	0.732[c]	1.17[c]				
Equol mg/day								
LP	3 990[a]	140[c]	30.4[c]	14.1[c]	165	< 0.001	< 0.001	< 0.001
SP	729[b]	16.6[c]	4.13[c]	1.25[c]				
FP	272[bc]	11.2[c]	3.63[c]	0.934[c]				
Prunetin mg/day								
LP	17.6[a]	< 0.267[c]	< 0.247[c]	< 0.323[c]	0.871	< 0.001	< 0.001	< 0.001
SP	6.16[b]	< 0.062[c]	< 0.062[c]	< 0.054[c]				
FP	< 0.274[c]	< 0.252[c]	< 0.273[c]	< 0.297[c]				
Matairesinol mg/day								
LP	1.62	1.49	< 1.28	1.67	0.330	< 0.001	0.422	0.966
SP	0.608	0.403	< 0.347	< 0.269				
FP	1.71	<1.41	< 1.36	< 1.58				
Secoisolariciresinol mg/day								
LP	< 2.72	< 2.83	< 2.61	< 3.42	0.430	< 0.001	0.353	0.612
SP	< 1.03	< 0.660	< 0.659	< 0.575				
FP	< 2.90	< 2.68	< 2.80	< 3.30				
Enterolactone mg/day								
LP	284	326	297	472	77.2	< 0.001	0.005	0.418
SP	241	164	196	244				
FP	437	396	499	654				
Enterodiol mg/day								
LP	3.77	3.79	2.94	3.82	0.857	< 0.001	0.632	0.859
SP	1.33	0.764	0.847	0.755				
FP	4.06	3.35	3.90	4.60				
Coumestrol mg/day								
LP	4.92[a]	5.23[a]	2.16[b]	< 1.77[b]	0.498	< 0.001	< 0.001	< 0.001
SP	1.46[b]	0.801[b]	0.451[b]	< 0.298[b]				
FP	< 1.51[b]	< 1.39[b]	< 1.50[b]	< 1.63[b]				

[a,b,c,d] Different letters within phytoestrogen indicates statistical difference within treatment and phase (*p<0.05).* SED= standard error deviation.

Flow of DM (Table 3) in omasum digesta mainly followed LP and FP with an average percentage distribution of DM passing per day on 46 for LP, 11 for SP and 43 for LP. A number of phytoestrogens was not detectable in omasum phases, and when calculating the distribution of phytoestrogens between phases, these will follow DM flow. Isoflavones and coumestrol mainly followed LP independent of diet, with 80.6 % (SD ± 7.8 %) presented in LP for the quantitatively most important isoflavones (biochanin A, formononetin and equol), and about 11.7 % (SD ± 2.2 %) presented in SP, and 9.0 % (SD ± 6.2 %) in FP. Daidzein differ slightly from the other isoflavones on RC and TI, where 60.3 % and 53.6 % followed FP and 29.4 % and 41.3 % LP for the respective diets. The dominating mammalian lignan, enterolactone, had a distribution where 32.7 % (SD ± 3.5 %) followed LP, 20.3 % (SD ± 3.3 %) followed SP and 47.1 % (SD ± 2.5 %) followed FP: Enterodiol followed DM greater than enterolactone, although enterodiol was over the detection limit, with 42.3 % (SD ± 4.1 %) presented in LP, 10.9 % (SD ± 2.9 %) in SP and 46.9 % (SD ± 4.2 %) in FP.

Metabolism of isoflavones

Metabolism of biochanin A and genistein is shown in Table 10.

Table 10. Metabolism of biochanin A and genistein (BG = biochanin A + genistein) for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI)

	Treatment					
	RC	BD	PR	TI	SED	p-value
Intake (BGi) mg/day	18 904[a]	792[b]	109[b]	42.9[b]	963	< 0.001
Digestive tract						
Omasum (BGo) mg/day	115[a]	3.10[b]	3.10[b]	3.59[b]	11.3	< 0.001
BGo/BGi mg/mg	0.006[b]	0.004[b]	0.036[b]	0.092[a]	0.014	0.003
Feces (BGf) mg/day	9.15	1.10	1.00	1.06	3.71	0.169
BGf/BGi mg/mg	< 0.001[b]	0.001[b]	0.011[b]	0.027[a]	0.004	0.001
BGf/BGo mg/mg	0.100[b]	0.372[a]	0.323[ab]	0.305[ab]	0.065	0.022
Intermediary						
Urine mg/day	0.444	0.321	0.207	3.26	1.12	0.091
Milk (BGm) µg/day	86.4[a]	51.0[b]	46.1[b]	28.8[b]	7.9	0.002
BGm/BGi µg/mg	0.005[b]	0.070[b]	0.531[a]	0.693[a]	0.128	0.004
BGm/BGo µg/mg	0.787[b]	17.1[a]	14.7[a]	8.27[ab]	3.24	0.009
Excreted						
Total excreted mg/day	9.68	1.47	1.25	4.35	4.11	0.244
Recovery (excreted/intake)	< 0.001[b]	0.002[b]	0.014[ab]	0.105[a]	0.027	0.022

[a,b] Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Intake, amount entering omasum and excretion in milk and feces of biochanin A and genistein was higher on RC than on other diets, but transfer rates to omasum and feces and apparent recovery in milk were the lowest. TI had the lowest intake and amount excreted in milk, while the highest transfer rate was from feed to omasum, feces and apparent recovery in milk. A high transfer rate from feed to omasum indicates a lower degree of metabolism in reticulo-rumen of biochanin A and genistein on TI.

Excretion of biochanin A and genistein to milk was significant higher on RC than on the other diets, about three times higher than on TI that had the lowest milk excretion. The apparent recovery from feed to milk was higher on the grass silage diets (TI and PR) than on the diets with herbs (RC and BD). The transfer rate from omasum to milk was higher on BD and PR than on RC and TI diets. There were no significant dietary effects on excretion through feces

and urine, or total excretion. Transfer rate from feed to feces was higher on TI than the other diets, while the transfer rate from omasum to feces was higher on BD than on RC.

Metabolism of formononetin and daidzein to their metabolite equol is shown in Table 11.

Table 11. Metabolism of formononetin and daidzein (FDE = formononetin + daidzein + equol) for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI)

	Treatment					
	RC	BD	PR	TI	SED	p-value
Intake (FDEi) mg/day	38 282[a]	2 709[b]	533[b]	121[b]	1767	< 0.001
Digestive tract						
Omasum (FDEo) mg/day	5 710[a]	194[b]	46.0[b]	18.9[b]	287	< 0.001
FDEo/FDEi mg/mg	0.149[a]	0.072[b]	0.090[b]	0.164[a]	0.015	0.002
Feces (FDEf) mg/day	1 196[a]	44.3[b]	14.2[b]	5.51[b]	105	< 0.001
FDEf/FDEi mg/mg	0.032	0.016	0.028	0.047	0.009	0.079
FDEf/FDEo mg/mg	0.213	0.233	0.314	0.272	0.051	0.300
Intermediary						
Urine mg/day	449[a]	105[b]	6.09[b]	16.9[b]	78.6	0.004
Milk (FDEm) µg/day	7 831[a]	964[b]	282[b]	101[b]	721	< 0.001
FDEm/FDEi µg/mg	0.204	0.349	0.515	0.854	0.228	0.116
FDEm/FDEo µg/mg	1,38	4.97	6.05	5.54	1.55	0.079
Excreted						
Total excreted mg/day	1 653[a]	150[b]	20.6[b]	22.5[b]	121	< 0.001
Recovery (excreted/intake)	0.043	0.056	0.039	0.233	0.059	0.045

[a,b] Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Metabolism of formononetin, daidzein and their metabolite equol followed the same pattern that biochanin A and genistein; intake was higher on RC than on other diets, but transfer rate milk were the lowest. TI had the lowest intake and amount excreted in milk, while the highest transfer rate was from feed to omasum, feces and milk.

Intake and amount of formononetin, daidzein and their metabolite equol entering omasum was significant higher on RC than the other diets, with 10-316 times higher intake and 30-302 times higher amount passing to the omasum. Metabolism of formononetin and daidzein in reticulo-rumen was lower for RC and TI than the other two diets. Milk excretion of

formononetin, daidzein and equol was higher for RC than the other diets, with decreasing amount with decreasing intake on the other diets. There was no difference in transfer rate from feed and omasum to milk between diets, but there was a tendency for increasing apparent recovery from feed to milk with decreasing intake. Excretion to feces, urine and total was significant higher on RC than on the other diets. There was no difference between diets in transfer rate from feed and omasum to feces. The overall recovery, from intake to total excreted was higher on TI than on the other diets.

Metabolism of lignans

The metabolism of secoisolariciresinol, mateiresinol, enterodiol and enterolactone are shown in Table 12.

Table 12. Metabolism of Secoisolariciresinol and Mateiresinol (SMEE = secoisolariciresinol + matairesinol + enterodiol + enterolactone) for red-clover diet (RC), botanical diverse diet (BD), perennial ryegrass diet (PR) and timothy diet (TI)

	Treatment					
	RC	BD	PR	TI	SED	p-value
Intake (SMEEi) mg/day	97.9^{c}	112^{bc}	151^{b}	214^{a}	11.9	< 0.001
Digestive tract						
Omasum (SMEEo) mg/day	982	903	1010	1 390	199	0.168
SMEEo/SMEEi mg/mg	10.0	7.93	6.73	6.55	1.26	0.105
Feces (SMEEf) mg/day	567	561	711	850	123	0.156
SMEEf/SMEEi mg/mg	5.75	4.97	4.73	4.05	0.663	0.186
SMEEf/SMEEo mg/mg	0.568	0.648	0.730	0.619	0.101	0.494
Intermediary						
Urine mg/day	4.92^{b}	106^{a}	121^{a}	98.8^{a}	7.92	< 0.001
Milk (SMEEm) µg/day	405	835	482	847	149	0.048
SMEEm/SMEEi µg/mg	4.11^{ab}	7.38^{a}	3.21^{b}	3.89^{ab}	1.17	0.044
SMEEm/SMEEo µg/mg	0.409^{b}	0.898^{a}	0.512^{b}	0.602^{ab}	0.105	0.016
Excreted						
Total excreted mg/day	573	668	833	950	128	0.092
Recovery (excreted/intake)	5.79	5.94	5.54	4.52	0.707	0.277

[a,b,c] Different letters within row indicates statistical difference between treatment (*p<0.05).*

SED= standard error deviation.

Metabolism of lignans differs from metabolism of isoflavones by higher content of lignans in omasum than intake and thus an apparently low metabolism in reticulo-rumen. Transformation from omasum to milk was highest on BD, with lowest content in omasum,

Intake of secoisolariciresinol and mateiresinol was significantly highest for TI diet and lowest for RC. However, no difference in omasal flow, rumen metabolism or in excretion to milk of lignans was found between diets. The apparent recovery from feed to milk transfer rate was higher on BD than on PR. The transfer rate from omasum to milk was also higher on BD and lowest for RC and PR. Diet had no effect on fecal excretion or on the transfer rate from feed to feces or omasum to feces. Urinary excretion of the sum of lignans was lower on RC than on other diets. For all diets excretion of lignans was much higher than the apparent intake, and there appeared no effect of diet on the overall recovery of lignans.

3.4 Discussion

3.4.1 Feed intake and milk yield

Milk yield was lower for TI, due to a slightly lower feed intake than the other treatments (Table 3). Timothy had, compared with the other silages, higher content of NDF and lower content of protein (Table 2), which may have caused the lower feed intake (Van Soest 1982b).

3.4.2 Concentration of phytoestrogens in feeds

Higher level of isoflavones in silage containing red clover was expected since red clover contains higher concentrations of isoflavones than grasses (Mustonen et al. 2009; Saloniemi et al. 1995; Wu et al. 2003).

The concentration and distribution between the different isoflavones in red clover silage was similar to the concentration Steinshamn et al. (2008) found in red clover silage (36 % red clover), except for biochanin A that was about 33 % (0.7 g/kg DM) lower in this experiment. Compared with other feeding experiment (Mustonen et al. 2009), the concentration of biochanin A, genistein, formononetin and daidzein in red clover silage, was 40-70 % (1.8-7.2 g/kg DM) lower in this experiment. Andersen et al. (2009) found concentrations of isoflavones in grass/clover silage much lower than red-clover silage in this experiment. This may be due to low content of red clover in their silage (2.6 %). Concerning botanical diverse silage the concentration was similar to those found by Andersen et al. (2009). Difference between experimental silages was expected, due to that environmental factors can affect the concentration of phytoestrogens in silages. Content of formononetin, biochanin A, genistein and daidzein is shown to be highest in young red clover, but is also affected by growing conditions such as water supply, nutrients supply, diseases and temperature (Kallela et al. 1987). Content of phytoestrogens is also shown to rise under silage ensiling (Sarelli et al. 2003).

Of the lignans, secoisolariciresinol was the dominating in all silages. This is consistent with the findings of Steinshamn et al. (2008), although the concentration of lignans was lower in all silages in this experiment. Higher content of secoisolariciresinol compared to matairesinol may be due to secoisolariciresinol being an intermediate in the synthesis of matairesinol (Dixon 2004). Concentration of lignans was higher in all silages than in the barley based

concentrate. This supports the suggestion from Steinshamn et al. (2008) that forage is more important than grain for the intake of secoisolariciresinol and mateiresinol.

A low concentration of coumestrol in silages was expected, in accordance with the results reported in previous studies (Andersen et al. 2009; Steinshamn et al. 2008). Steinshamn et al. (2008) found a higher concentration of coumestrol in white clover silage than in red clover silage. This is consistent with a higher concentration in botanical diverse than red clover silage in this experiment.

3.4.3 Metabolism of phytoestrogens

Rumen metabolism of phytoestrogens

According to Lundh (1995) the major metabolic transformation of isoflavones is performed by rumen microbes, and a high transformation in rumen was expected. In this experiment only 0-9 % of daily biochanin A and genistein intake was found in omasum (Table 10). 7-16 % of daily formononetin and daidzein intake was found in omasum, mainly as the metabolite equol (Table 11). This shows a high degradation and metabolism of isoflavones in the rumen, and confirms what Lundh (1995) found. For biochanin A and genistein the transfer rate from feed to omasum decreased with increasing intake (Table 10). TI had the lowest intake and the highest transfer rate, while RC had the highest intake and the lowest transfer rate of biochanin A and genistein. Rumen microbes adapt to the content of phytoestrogens in the feed, and will become more efficient in metabolizing phytoestrogens over time. After 6-10 days rumen microbes will be adapted, and biochanin A and genistein will almost be completely metabolized to *para*-ethyl phenol and organic acids without estrogenic effect (Seested et al. 2000; Shutt et al. 1970). A lower metabolism with decreasing intake could be due to lower degree of adaptation. The same was partially shown for formononetin, daidzein and equol, with the lowest metabolism for TI diet (Table 11), and an increasing metabolism when increasing intake. RC differs from this, which may be due to limiting factors in microbial metabolism in reticulo-rumen, with high concentration of precursors. *In vitro* study with incubation of formononetin and biochanin A in bovine rumen fluid showed that half-life for formononetin was 4.3 hours, for daidzein: 9.3 hours, for biochanin A: 3.9 hours and for genistein: 5.5 hours (Dickinson et al. 1988). Long half-life of formononetin and daidzein could explain why a higher percentage of those isoflavones, including equol, passed to omasum.

Content of lignans in omasum and the excreted amount of secoisolariciresinol, matairesinol, enterodiol and enterolactone was many times higher than intake of secoisolariciresinol and matairesinol. This indicates that other lignans may be metabolized to enterodiol and enterolactone than those measured in this study. In their study of *in vitro* of lignan metabolism in human fecal inoculum, Heinonen et al. (2001) presented syringaresinol, pinoresinol, lariciresinol, arctigenin and 7-hydroxymatairesinol as precursors for enterodiol and enterolacton. Pinoresinol and lariciresinol is precursors in the biosynthesis of secoisolariciresinol and matairesinol, and the intestinal microbial metabolism of pinoresinol seems to occur in a similar way, with lariciresinol and secoisolariciresinol as intermediates (Dixon 2004; Heinonen et al. 2001). To our knowledge these precursors are not analyzed for in grass or silages before. It is known that lignans are mainly found in the fiber layers in plants (Petit et al. 2009; Tham et al. 1998). Begum et al. (2004) showed their experiment with rats that lignin is an precursors for lignans, and due to this it could be expected that diets containing most NDF had highest excretion of lignans. In the present study, TI had the highest intake of NDF, content of lignans in digesta passing to omasum, and amount excreted.

Omasal flow of phytoestrogens

To our knowledge content of and passage of phytoestrogens to omasum in dairy cows have not been reported before.

Equol was the dominating isoflavone in the digesta on all diets, followed by formononetin in RC, BD and PR. Equol being dominating was expected and consistent with previous experiment (Shutt et al. 1970). Equol was the dominating isoflavone in rumen and abomasal digesta when sheep's where fed two sorts of clover (Shutt et al. 1970). Dickinson et al. (1988) incubated formononetin in bovine rumen fluid, and found that 24-hour incubation resulted in almost complete demethylation of formononetin to daidzein and rapid conversion of daidzein to equol. Passage of formononetin to omasum may be due to that some phytoestrogens avoid being metabolized if the passage rate of DM from rumen is higher than metabolism of phytoestrogens. Other possible explanations could be limitations in demethylation of formononetin.

Table 7 shows that all the different isoflavones was further metabolized or absorbed in the intestines, and due to the relatively low recovery of isoflavones in milk and urine there must be a considerably metabolism in the gut or intermediary. Recovery of biochanin A and

genistein (Table 10) was below 1 % for RC and BD, 1 % for PR and 10.5 % for TI. For formononetin, daidzein and equol the recovery was 4-6 % for RC, BD and PR, and 23 % for TI. The highest recovery was for the diets whit the lowest intake, which could be explained by lower adaption of microbes when low concentrations of biochanin A and genistein.

Enterolactone was the dominating lignan in omasum digesta, followed by enterodiol for all diets. This was expected due to previous *in vitro* study; Côrtes et al. (2008) carried out an study on metabolism of lignans in ruminal and fecal inoculum, and showed that metabolism of secoisolariciresinol first led to production of enterodiol in rumen, which further was converted to enterolactone.

Isoflavones mainly followed LP when passing to omasum, while lignans mainly followed LP and SP where the majority of DM was presents (Table 9). This was not expected since phytoestrogens is water soluble and, therefore, should be found in FP. An explanation of this could be that phytoestrogens are located inside or nearby rumen microbes attached to particles. Lignin is an precursor for lignans (Begum et al. 2004), and due to this it can be possible that mammalian lignans is located near the lignin, in-or nearby microbes that ferments NDF. NDF consist of cellulose, hemicellulose and lignin (Van Soest 1982a). For further studies it may be interesting to isolate microbes from rumen or omasum and analyze those for phytoestrogens to determine whether mammalian phytoestrogens are located inside microbes, or follows plant residues.

Metabolism of phytoestrogens in the intestines

Domination of equol in feces was expected and in accordance with Tucker et al. (2010) who found that when isoflavone intake increased, excretion in feces increased in 5 out of 7 measured isoflavones. The amount excreted isoflavones was lower for the experiment of Tucker et al. (2010) due to a lower intake of isoflavones. The amount of isoflavones excreted in feces per day in this experiment, was lower than amount passing to omasum, indicating that isoflavones were further metabolized or absorbed in the intestines.

A higher rumen metabolism of lignans than metabolism in the intestines (Table 12) supports the findings of Gagnon et al. (2009a). He reported that the main site of metabolism of flax lignans in dairy cows is the rumen, and that the metabolism in the intestines is not that efficient. From this experiment, it appears that the result from Gagnon and Coworkers also is true for other diets. Results from this experiment also shows that the amount of lignans

excreted in feces, urine and milk was lower than the amount passing to omasum, indicating that an amount of lignans was absorbed or further metabolized in the intestines to other not detectable compounds.

Net production of enterodiol on PR and TI in the intestines was expected due to previous findings, although enterolactone was the dominating excreted lignan in feces (Côrtes et al. 2008). Côrtes et al.(2008) showed in their *in vitro* study of metabolism of lignans by ruminal and fecal microbes that net production of enterolactone by ruminal microbes was higher than of enterodiol while net production of enterodiol was higher than enterolactone when incubating secoisolariciresinol in fecal microbes, suggesting that the main mammalian lignan produced by fecal microbes from secoisolariciresinol-diglucoside is enterodiol. Our result for PR and TI supports this, while we have no good explanation why there was a decrease in enterodiol on RC and BD.

Intermediary metabolism of phytoestrogens and concentration in milk

Transfer rate to urine will in a small extent be discussed, due to insecure analyses.

Concentration of isoflavones in milk was effected of the concentration in feed. With increasing concentration in feed the concentration in milk was also increasing. This is partially supported by previous findings (Andersen et al. 2009; Steinshamn et al. 2008).

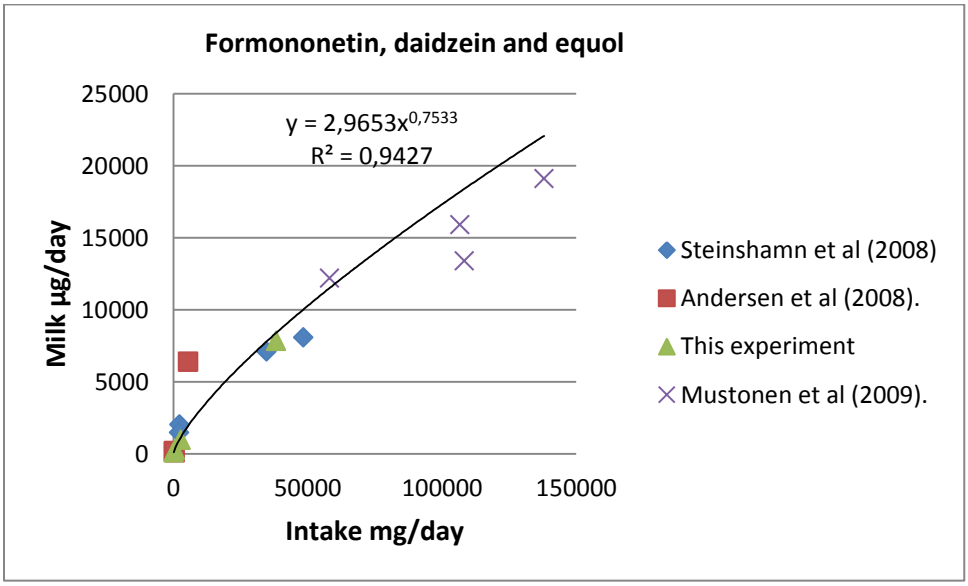

Figure 1. Summary of literature for 16 treatment means showing the effect of increasing the intake of formononetin and daidzein (mg/day) on content of formononetin, daidzein and equol in milk (µg/day).

It appears from both this experiment (Table 10 &11) and other experiments (Figure 1) that the amount of isoflavones excreted in milk are closely related to the intake, especially the excretion of equol and the intake of its precursors. Milk excretion of biochanin A and genistein increased also with intake in this experiment, but to a less extent. Due to the high metabolism of these compounds, little opportunities to influence the content in milk is to be expected (Seested et al. 2000). It seems to be less opportunity to affect the excretion of lignans in milk (Table 12). However, as there are obviously other plant lignans that are more important precursors to enterolactone and enterodiol than secoisolariciresinol and matairesinol, it is not possible to be conclusive. More research is needed in order to find this precursor lignans.

Equol, which is a metabolite of the metabolism of formononetin and daidzein, was the dominant isoflavones in milk from all diets, and is also one of the most investigated isoflavone. The concentration of equol in milk from RC were similar to the concentration found in other feeding experiments with red-clover silages; Steinshamn et al. (2008) found an concentration of 364 µg/l, while Mustonen et al. (2009) found from 410-695 µg/l. Antignac et al. (2004), Hoikkala et al. (2007) and Purup et al. (2005) compared equol organic and conventional milk; conventional milk contained 36.4, 61.6 and 41.0 µg/l respectively, and organic milk contained 191, 410.9 and 230 µg/l respectively. A high concentration of isoflavones in organic milk was due to that diet for organic held dairy cows often contains more legumes than conventional diets. The concentration in conventional milk from the three studies is comparable to the concentration of equol in BD, PR and TI diet from this experiment.

Apparent recovery of isoflavones from feed to milk (Table 10 & 11) has been reported by Steinshamn et al. (2008) and Andersen et al. (2009). Recovery of formononetin, daidzein and equol on BD in this experiment was lower than expected from previous studies. This applies also for biochanin A and genistein on TI, which was lower than expected based on the recovery found by Andersen et al (2009) at similar intake. Apparent recovery of isoflavones from feed to milk was highest on diets containing the lowest amount of isoflavones, which is consistent with the results found by Steinshamn et al. (2008) and Andersen et al. (2009) (Figure 2 and 3).

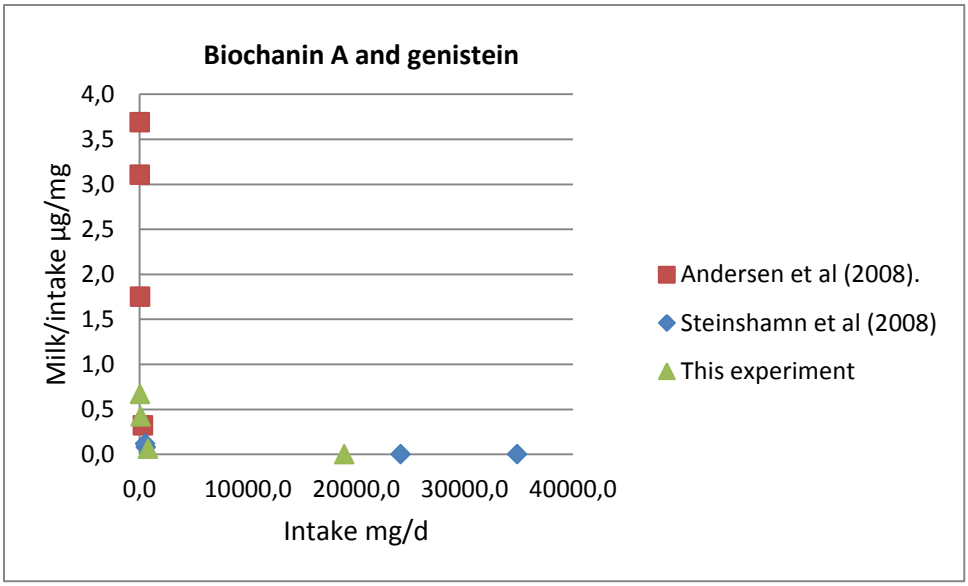

Figure 2. Summary of literature for 12 treatment means showing apparent recovery of biochanin A and genistein from feed to milk.

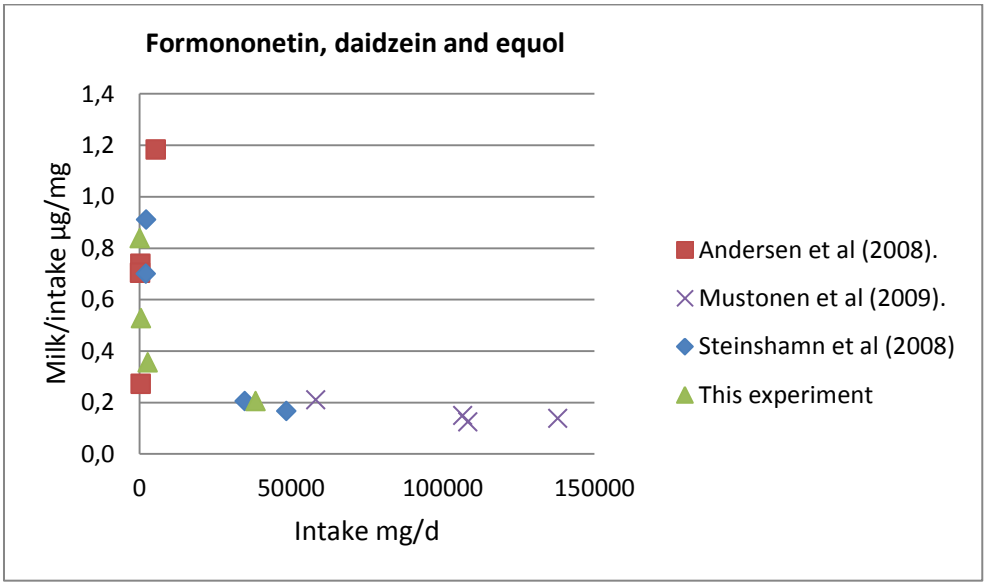

Figure 3. Summary of literature for 16 treatment means showing apparent recovery of formononetin, daidzein and equol from feed to milk.

Enterolactone was the dominating lignan, with the highest concentration in milk from timothy silage. Concentration in milk from timothy and botanical diverse silage was similar to the level reported in previous studies (Antignac et al. 2004; Gagnon et al. 2009b). Steinshamn et al. (2008) found a concentration of 27.0 µg/l in milk from white clover silage, and 21.6 in milk from red clover silage, which was comparable to the concentration in milk from RC (17.9 µg/l) and PR (23.3 µg/l) in this experiment.

Apparent recovery of lignans in milk was, even with an intake at the same level, lower than found by Steinshamn et al. (2008) and Andersen et al. (2009) found. There was a low recovery on diets with a high intake, which agrees with previous experiments (Figure 4), although not as pronounced as for isoflavones (Figure 2 & 3). A reason for this could be that unknown lignans as precursors for enterodiol and enterolacton exists in various amounts, and thus the estimation of intake could be too low.

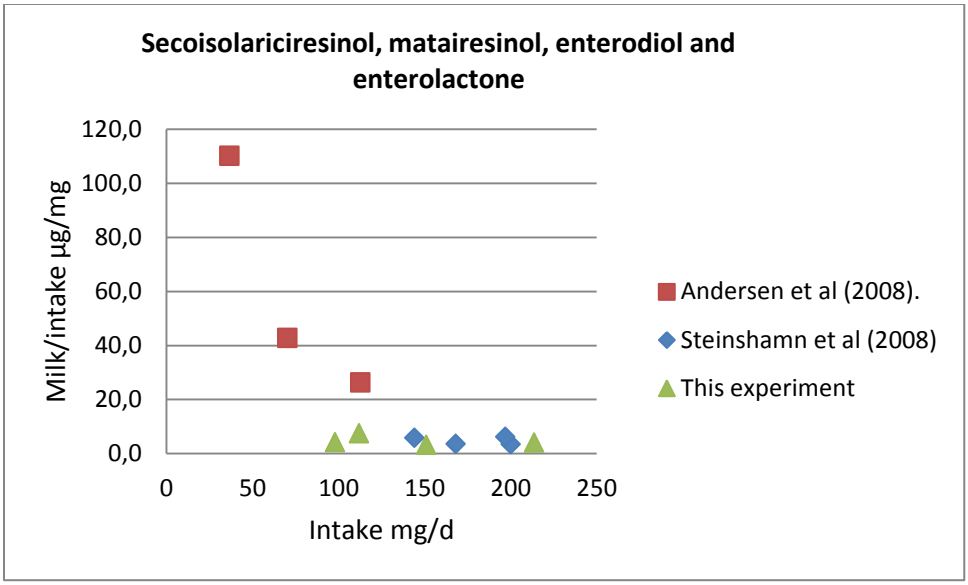

Figure 4. Summary of literature for 11 treatment means showing apparent recovery of secoisolariciresinol, matairesinol, enterodiol and enterolactone from feed to milk.

From the figures (1, 2, 3 &4) it appears that excretion of phytoestrogens in milk (in percentage of intake) is decreasing with increasing intake. Steinshamn et al. (2008) suggest explanations for this; metabolism in the gastrointestinal tract and the hepatic reconjugation before blood transport and secretion to the udder could be rate limiting, and epithelial cells in mammary gland might be only permeable to estrogenic compounds. From the results in this experiment, with a high metabolism in rumen and gastrointestinal tract, the limitation appears to be intermediary. Enzymatic reactions follow 1st order Michaelis-Menten kinetics, with a decreasing product with increasing substrate. Active transport of substrates from blood to tissue is also expected to follow this kinetics (Madsen & Nielsen 2003), so it is possible that both reconjugation and secretion to cells in udder could be the limiting factor.

3.5 Conclusions

Phytoestrogens are extensively metabolized in the rumen to mammalian phytoestrogens and unknown compounds. Metabolism of isoflavones was dependent of intake, with the lowest recovery in omasum on diets with the highest concentration and intake of isoflavones. Isoflavones and coumesterol mainly passed to omasum with large particles, while mammalian lignans where evenly distributed between phases when passing to omasum. Total apparent recovery of mammalian lignans was higher than the intake of measured plant lignans. This was probably due to a high amount of unknown precursor lignans in the diet. Concentration of phytoestrogens in milk can be manipulated trough diet; concentration of phytoestrogens in milk increased with increasing concentration in feed. However; apparent recovery of isoflavones in milk decreased with increasing intake, and for lignans the apparent recovery from omasum to milk showed the same pattern. It appears to be intermediary factors that limit full transition to milk.

3.6 Acknowledgments

Thanks to the CORE Organic Funding Body Network for funding the research project, and TINE for financial support for this master thesis.

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