Effect of litter size and bacitracin administration on tissue protein synthesis of lactating rabbit does.

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Running head

Protein synthesis in lactating does fed bacitracin

Abstract

Bacitracin is an antibiotic used in rabbit husbandry to control microbial digestive pathologies. Collateral effects on absorption and mucosal development have been reported and these may impact on protein metabolism. The present study aims to analyse the effect of the antibiotic on protein synthesis in lactating does because mammary gland metabolism and milk output should provide a sensitive index of any undesirable action of bacitracin. Rates of protein synthesis were measured in mammary gland, liver, intestinal mucosa and muscle of lactating rabbits does by injecting a flooding dose of [$^{2}H_5$]phenylalanine into the auricular artery of two groups (each n=8) of New Zealand White does fed different experimental diets. The control group (C) received the basal diet and the bacitracin group (B) ingested the same diet but supplemented with bacitracin (100 mg/kg). Animals received the experimental diet from d 28 of pregnancy until d 26 of lactation when they were slaughtered. Just after birth, litter size (LS) was adjusted by cross-fostering either to 5 or 9 pups (4 does per dietary treatment). The relative weight of the liver tended to be greater in those females receiving the B diet (27 *vs* 22.5 g/kg BW; $P<0.07$), while diet did not effect mammary gland weight (255.7 ± 10.59 g). Fractional protein synthesis rate (FSR) was higher for intestinal mucosa (duodenum; 51.7 ± 2.09 %/d) followed by mammary gland and liver (38.29 ± 2.62 %/d and 40.2 ± 1.98 %/d, respectively), and the lowest value was observed in muscle (2.92 ± 0.26 %/d; $P<0.0001$). Bacitracin treatment lowered FSR in the mammary gland by 23% ($P = 0.024$) and this was independent of litter size. Conversely, FSR in the duodenum was not affected by antibiotic treatment but reduced by 15% ($P = 0.021$) for the larger litter size.

Keywords

Protein Synthesis, Mammary Tissue, Liver, Rabbits does

Implications

Bacitracin, as feed additive, has been banned from livestock diets (EU 1831/2003) because of the widespread use would increase pathogen resistance. Moreover bacitracin is not innocuous in metabolic terms given that altered protein metabolism observed here for lactating does.

Introduction

Zinc bacitracin is the most common antibiotic used in rabbit husbandry to control digestive pathologies induced by intestinal micro-organisms, although collateral effects on nutrient absorption (Abecia *et al.,* 2005) or intestinal mucosa development (King, 1980) have been also described. Bacitracin is a polypeptide antibiotic, produced by *Bacillus licheniformis,* that acts by interfering with the formation of lipid-linked sugars and thus disrupting the synthesis of peptidoglycan in bacterial cell membranes (Storm and Strominger, 1973 and 1974). These lipid-linked sugars are intermediates in the biosynthesis of several glycoproteins and polysaccharides not only in bacteria (Waechter and Lennarz, 1976) but also in plants (Ericson *et al.*, 1978) and animals (Herscovics *et al*., 1977). Moreover, bacitracin has been shown to impact on both isolated cells and subcellular organelles, through inhibiting the degradation of substances that influence protein synthesis, including thyrotropin, luteinising hormone releasing factors (McKelvy *et al.*, 1976), insulin (Roth et al., 1980) and β-endorphin (Patthy *et al.*, 1977). Collateral effects of bacitracin on nutrient absorption (Abecia *et al*., 2005; King, 1980) may be exerted through protein metabolism, either by blocking synthesis of specific cell wall peptidoglycane or altering the hormonal environment of protein metabolism. During lactation, protein metabolism is enhanced (Baracos *et al.*, 1991) due to the metabolic activity of the mammary gland and thus may be particularly sensitive to any undesirable action of the antibiotic.

The aim of this study was to investigate the impact of the antibiotic on protein synthesis in lactating does using litter size as a tool to modify intake.

Material and methods

Animals and diets

Protocols, animal handling and infusion procedures used in this experiment were approved by the Comité Etico de Experimentación Animal of the University of Zaragoza. Sixteen New Zealand White doe rabbits in the third pregnancy and a similar body weight, (4.3 ± 0.42 Kg) at the beginning of the experiment were randomly divided into two groups that were fed from late pregnancy (2-3 d before parturition). Ingredients and chemical composition of experimental diets were described on Table 1.

Experimental design

Less than 12 hours after birth, the litter size of four does within each dietary treatment was adjusted by cross-fostering either to 5 (LS5) or 9 (LS9) pups. The females were housed separately from their offspring in individual metabolism cages and pups were allowed to suckle for 10 min every morning. Daily milk yield was recorded by weighing the does immediately before and after suckling. On day 26 after parturition the does were weighed, a manual milk sample (5-10 ml) obtained and then they were suckled by their offspring. Afterwards the females were fitted with two indwelling catheters: one in the auricular artery [20G $1^{1/4}$ ″;1.1 x 32mm] and the other in the marginal vein [22 G 1″; 0.9 x 25mm; Braun Medical S.A, Rubi, Barcelona) of the contralateral ear, for infusion and sampling, respectively. Surgery was performed under sterile conditions in an appropriately equipped operating room. To insert the catheters, the ears were anesthetised using a commercial topical cream (EMLA, Astra-Zeneca Farmaceutico S.A, Madrid, Spain).

Infusion protocol and tissue sampling

A flooding dose of phenylalanine (set at 15 times the size of the body free phenylalanine pool), 40 % of which was as [ring-$^{2}H_5$]phenylalanine (Cambridge Isotope Laboratories IL, Inc., Miamisburg, OH, USA), dissolved in sterile saline (9g NaCl/l) was infused over a 10 min period into the artery. The amount of total phenylalanine infused per doe averaged 400 mg. Venous blood samples (1 ml) were withdrawn at -10 min (for background natural abundance) and at 12, 15, 20, 25, 30 and 40 min after the start of the infusion. Therefore, isotope was infused and sampled during 40 min in order to obtain an equilibrium of the isotope distribution into the plasma pool but avoiding the return of bounded-[$^{2}H_5$] phenylalanine. These samples were used to define the temporal kinetics of plasma free phenylalanine enrichment. After the last plasma sample a small volume of milk (2-5 ml) was taken manually and then does were killed by lethal injection of sodium thiopental (Braun Medical S.A, Rubi, Barcelona). In order, tissue samples (2–3 g) from the liver, *semitendinosus* muscle, mammary gland and duodenum were rapidly dissected, washed in cold saline and frozen in liquid nitrogen until analysis. The remainder of the liver and mammary gland were then extracted and weighed.

Analytical procedures

Dry matter (DM), organic (OM) matter, nitrogen (N) and fibre in feed and faeces and N in urine were determined by standard procedures of AOAC (Association of Official Analytical Chemists, 1995). Casein in defatted milk samples (2 ml) was determined after cool-casein coagulation using chymosin (CHR Hansen, Madrid, Spain) and calcium chloride followed by centrifugation (15000 g, 1h at 4ºC). N was determined in both the supernatant (serum protein) and casein-precipitated fractions.

Isotope determination

Free phenylalanine extracts from both plasma, obtained from centrifugation of blood at 1000 g for 15 min at 4°C, and tissues were obtained, converted to t-butyldimethsilyl derivatives and isotopic enrichments measured by gas-chromatography mass spectrometry (GCMS) as described previously (Connell *et al*., 1997) and calculated as mole per cent excess (MPE). Protein-bound phenylalanine from the tissues was enzymatically converted to phenylethylamine and the enrichments determined as the heptafluorobutyryl n-butyl ester as described previously (Calder *et al*., 1992).

Calculation and statistics

Fractional rates of protein synthesis (FSR; percentage of the tissue protein pool synthesised per day) in the different tissues were calculated using the equation developed by Garlick *et al.,* (1980) for the "flooding" dose technique.

$$FSR\ (\%/d) = (S_b / S_a)*(100/t)$$

Where *Sb* is the isotopic enrichment of the phenylalanine bound to tissue protein above natural abundance (assumed to be the same as in plasma protein) at time t (d^{-1}), the time of tissue removal. *Sa* is the calculated area under the curve (AUC) for the isotopic enrichment of the free phenylalanine pool, between time = 0 until t, calculated by trapezium-based analysis for each doe and tissue. The AUC was calculated from the plasma data, extrapolated to the time of tissue excision, and this then corrected by the ratio of the terminal tissue free pool:extrapolated plasma phenylalanine enrichments.

Absolute rate of protein synthesis (ASR, g/d) in liver and mammary gland was calculated according to the equation:

$$ASR = (FSR/100) * \text{total protein content in tissue (g)}$$

Results were examined by ANOVA as a 2 x 2 factorial design, considering the diet (C *vs* B) and the litter size (LS5 *vs* LS9) as main effects. When comparisons between tissues

FSR were performed, rabbit was taken as a random factor. All statistical analyses were carried out using the GLM Procedure of the Statistical Analysis Systems computer software package, version 8 (SAS Institute Inc. 2000). Significance was taken as $P<0.05$.

Results

No major incidents were registered during the experiment and no apparent differences in behaviour were observed among does eating either the control or the medicated diet.

Production data from the last day of experiment (to match with isotope kinetics) are shown in Table 2. On the terminal day no treatment differences were observed for body weight (4011 (SE 40.35) g), dry matter intake (293 (SE 13.57) g/d) or milk yield (242 (SE 17.98) g/d). In addition, no significant changes were detected in either protein (107.7 g/Kg of milk) or casein (57.6 g/Kg) concentration in milk between experimental treatments, the latter constituted 54 % of total milk protein.

The relative and absolutes weights of the mammary gland averaged 63.8 (SE 2.24) g/Kg BW and 255.7 (SE 14.38) g, respectively, and were neither influenced by diet nor litter size. In contrast, the relative weight of the liver tended to be greater in those females receiving the B diet (27 *vs* 22.5 g/kg BW; P<0.07). Liver weight was independent of litter size.

Temporal changes in plasma enrichment of free phenylalanine are presented in Figure 1, together with terminal enrichments of free phenylalanine recorded for the different tissues. The FSR for the different tissues are presented in Table 3. The highest mean values were found for the intestinal mucosa (duodenum; 51.7 %/d) followed by mammary gland and liver (38.29 and 40.2 %/d) as showed in table 3. Protein contents in muscle, mammary gland and liver were 17.0 (SE 0.68), 15.2 (SE 1.05) and 15.8 (SE 0.57) g CP/100 g wet tissue, respectively. Bacitracin lowered FSR in the mammary gland by 23% (P = 0.024) and this was independent of litter size. Similar changes were observed when ASR was

considered. In contrast, protein synthesis in the duodenum was not affected by antibiotic treatment but was reduced (- 15%, *P*=0.021) for the larger litter size. No significant changes in FSR or ASR were detected for liver or muscle. Data from N balance are presented in Table 4. Feed N input (g/d) averaged 7.9 (SE 0.29) whereas output in urine (3.26 (SE 0.13)), faeces (2.12 (SE 0.16)) and milk (4.54 (SE 0.24)) totalled 9.92 g/d, so the does were in a negative daily balance of 1.9 g/N, equivalent to 12.1 g/protein, even at this stage of late lactation.

Figure 1 shows enrichment (mpe) of free phenylalanine in plasma. Liver was the first tissue sampled followed by muscle due to the relatively easy access to both of them. However to take mammary gland and duodenum samples longer time was consumed.

Discussion

The experimental animals formed part of a larger study (Abecia *et al*., 2008) but experimental constraints, including availability of metabolic cages, meant that only 16 does could have kinetics measurements performed. The limited numbers meant that some production data (discussed below) did not show the treatment differences reported earlier (Abecia *et al*., 2008). Nonetheless, the main objective focused on protein metabolism in responses to bacitracin was demonstrated.

Diet inclusion of bacitacin reduced FSR and ASR in the mammary gland and tended to increase the relative weight of the liver. Bacitracin intake has been shown not to affect either the caecotrophy process (Abecia *et al*., 2008) or the bacterial caecum population (Abecia *et al*., 2007) and, therefore, the responses in mammary protein synthesis and liver weight probably reflect the direct effect of bacitracin on metabolism of the doe.

During lactation, protein synthesis is crucial not only in the mammary gland but also in those organs involved in nutrient supply to the gland (e.g. liver and gut mucosa). All these tissues demonstrate higher protein synthesis rates during lactation (Baracos *et al*., 1991)

and thus may be particularly sensitive to modifiers of protein synthesis, including bacitracin.

The flooding-dose method proposed initially by Garlick *et al.* (1980) is suitable for short-term measurements in high turnover tissues or longer term analyses of slower turnover tissues. Ideally, at the end of the flood dose period the enrichments of free phenylalanine in plasma and the tissue should be similar so that the plasma dynamics can be extrapolated confidently to the tissues. As shown in Figure 1, this is the case for muscle and, to a lesser extent, for the mammary gland (final values 8% lower than plasma). Terminal values for liver and duodenum were, respectively, 25 and 31 % lower than plasma, however, due to dilution of the labelled phenylalanine by amino acid released by protein degradation within the tissue. For these tissues FSR calculated based on the plasma AUC would be under-estimated while values corrected for the terminal tissue value may be over-estimated.

Liver weight was higher in does fed on bacitracin but hepatic FSR was unaffected by bacitracin. The heavier liver may be due to a reduced fractional degradation rate of constitutive hepatic protein or, alternatively, be related to bacitracin effects in lipolytic activity, as demonstrated *in vitro* (Heckemeyer *et al.*, 1982). The authors are not aware of data on the effect of bacitracin on liver FSR but the values in the present study and the relationship between tissues agrees well with literature reports related to rabbits (Nicholas *et al*., 1977), rodents (Garlick *et al*., 1973) and ruminants (Attaix *et al*., 1988). The greater hepatic FSR (40.2 %/d) compared with younger rabbits (14.8 - 31.7 %; Nicholas *et al*., 1977) is probably technique related since the flood dose technique would incorporate hepatic export proteins while these would be mainly excluded from the continuous infusion approach (Nicholas *et al*., 1977). Export proteins have been proposed to account for approximately 30% of liver synthesis (Waterlow, 1991). In addition, lactation causes an increase in the whole-body protein turnover, including across the mammary gland, liver

and gastrointestinal tract (Millican *et al*., 1987), with hepatic metabolism elevated to provide more nutrients and protein for milk production.

Bacitracin intake did not modify muscle FSR. The current values (2.92 %/d) are similar to those reported previously for adult rabbits, using either a decay protocol (Signoret *et al*., 1973) or a continuous infusion method (Nicholas *et al*., 1977). Higher values (5 and 10 %/d, respectively) have been observed for young males of either up to 1.4 kg (Palmer *et al*., 1980) or 2-2.5 kg body weight (Laurent, 1982). FSR is known to be closely related to animal development (Laurent, 1982) and decreases with age (Millward *et al*., 1975; Lobley 1993) and the multiparous does used in this study are considered adult animals. Lactation has a clear effect on mammary and liver tissues (Sampson *et al*., 1986), but effects on muscle are not yet confirmed. In other species, muscle protein synthesis (FSR) can even be reduced in order to redirect amino acids toward those tissues involved in milk precursor synthesis (Baracos *et al*., 1991).

Protein synthesis within the mammary gland comprises mainly export protein, including caseins and the whey acidic proteins (WAP), plus minor endogenous tissue turnover. In addition, some proteins are synthesised in other tissues (mainly liver) and then transported via plasma to the mammary gland. The gland synthesises mostly α_{s1}-casein and β-casein plus some other proteins, relevant in ruminants but of minor importance in lagomorphs (Grabowski *et al*., 1991). The absolute synthesis of protein (15.06 g/d; Table 3) exceeds the secreted casein (13.9 g/d) but when allowance is made for WAP, reported to comprise 10% of total protein synthesized in milk (Grabowski *et al*., 1991), then much of the synthesis can be accounted. In support of previous reports in other species (Sampson *et al*., 1986; De Santiago *et al*., 1991), the mammary gland had a higher FSR than liver. Nonetheless, mammary gland FSR was not affected by litter size but, in practice, this did not lead to a significant change in daily milk yield at this late stage of lactation, but rather

the amount of milk available to each pup altered. Under condition of unchanged milk output there would be no need to increase the rate of protein synthesis. Interestingly, however, bacitracin decreased mammary gland protein FSR. As there were also no changes in the size of the mammary gland this meant that absolute synthesis was also decreased (P<0.03). Although this was not accompanied by any significant changes in milk volume output during the complete lactation cycle (Abecia *et al*., 2008), there were numerical changes in both protein and casein yield (Table 2) in the period just prior to the kinetic measurements and these were of comparable magnitude to the reduced protein synthesis. The mechanism behind such action remains obscure. Enhanced sensitivity compared with other tissues is one possibility, as are impacts on hormonal regulation, especially as a known action of bacitracin is the inhibition of degradation of a number of polypeptide hormones, including insulin, linked in some cases to reduced internalisation within cells (Bonser *et al*., 1983) and this may lead to repartitioning of nutrients away from the mammary gland.

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Table 1. Ingredients (g per kg of dry matter) and chemical composition (g per kg of dry matter) of both experimental diets.

Ingredients (g/kg of dry matter)	Control	+Bacitracin	Chemical composition (g/kg of dry matter)	Control	+Bacitracin
Grass hay	400	400	Organic matter	92,12	91,97
Wheat grain	200	200	Crude protein	19,06	18,61
Soyabean meal	150	150	Acid-detergent fibre	19,52	19,55
Barley grain	130	130	Neutral-detergent fibre	31,91	33,42
Sugarbeet pulp	100	100	Acid-detergent lignin	4,81	4,45
Sunflower-seed oil	5	5	Ether extract.	2,67	2,68
Ammonium sulfate	5	5			
Vitamin–mineral mix	10	10			
Bacitracin	-	0.1			

Vitamin mineral mixture composition: Co ($CoSO_4.7H_2O$), 200 parts per million (ppm); Cu ($CuSO_4.5H_2O$), 3000 ppm; Fe ($FeSO_4.H_2O$), 20 000 ppm; Mn (MnO_2), 8000 ppm; Zn (ZnO), 30 000 ppm; Se (Na_2SeO_3), 30 ppm; I (KI), 500 ppm; vitamin A, 270 mkat (4 500 000 IU)/ kg; vitamin D3, 33 mkat (550 000 IU)/kg; vitamin E, 1100 ppm; vitamin B1, 250 ppm; vitamin B2, 1500 ppm; vitamin B6, 100 ppm; vitamin B12, 6000 ppm; vitamin K, 500 ppm; Dpantothenate, 5000 ppm; niacin, 12 500 ppm; choline chloride,100 000 ppm.

Table 2. Body weight (g), dry matter intake (g/d), milk yield (g/d), protein yield and casein yield (g/d) of 16 does on the last day of the experiment (slaughter day) together with liver and mammary gland contribution to final body weight in lactating does fed on a conventional diet either supplemented (+bacitracin) or not (control) with zinc bacitracin (100 ppm) and nurturing 5 or 9 pups (SE for diet comparison).

		Diet			2-way ANOVA		
Last day (slaughter)	**Litter Size**	**Control**	**+Bacitracin**	**SE**	**Diet**	**LS**	**D x LS**
Body weight g	**9 pups**	4093	3924	44.5	NS	NS	NS
	5 pups	4052	3970				
DM intake g/d	**9 pups**	279	269	29.8	NS	NS	NS
	5 pups	313	310				
Milk Yield g/d	**9 pups**	260	233	42	NS	NS	NS
	5 pups	252	224				
Casein yield g/d	**9 pups**	15.0	13.5	2.42	NS	NS	NS
	5 pups	14.5	12.9				
Protein yield g/d	**9 pups**	28.0	25.2	4.52	NS	NS	NS
	5 pups	27.2	24.1				
Relative tissue weight g/Kg BW							
Liver	**9 pups**	22.0	29.3	1.54	0.07	NS	NS
	5 pups	23.1	24.6				
Mammary gland	**9 pups**	64.0	59.6	3.49	NS	NS	NS
	5 pups	65.8	65.7				

NS, *P*>0.05; SE, standard error; LS, litter size; D, diet.

Table 3. Fractional synthesis rates (FSR) in liver, duodenum, mammary gland and muscle together with absolute synthesis rates (ASR) of liver and mammary gland in 26d lactating does fed on a conventional diet either supplemented (+bacitracin) or not (control) with zinc bacitracin (100 ppm) and nurturing 5 or 9 pups (SE for diet comparison).

		Diet			2-way ANOVA		
	Litter Size	Control	+Bacitracin	SE	Diet	LS	D x LS
FSR (%/d)							
Liver	**9 pups**	40.3	41.4	1.98	NS	NS	NS
	5 pups	39.5	40.2				
Mam. Gland	**9 pups**	40.9[a]	33.8[b]	2.62	*	NS	NS
	5 pups	46.5[a]	32.8[b]				
Duodenum	**9 pups**	45.1[b]	49.7[b]	2.09	NS	*	NS
	5 pups	58.5[a]	53.5[a]				
Muscle	**9 pups**	3.14	3.22	0.26	NS	NS	NS
	5 pups	2.62	2.75				
ASR (g/d)							
Liver	**9 pups**	6.08	6.03	0.58	NS	NS	NS
	5 pups	6.38	6.01				
Mam. Gland	**9 pups**	15.88[a]	12.80[b]	1.50	*	NS	NS
	5 pups	20.48[a]	12.72[b]				

In a same line, means with different superscript differ ($P<0.05$)

NS, P>0.05; *, P<0.05; SE, standard error; LS, litter size; D, diet.

Table 4. Nitrogen balance in lactating does (d17 to d24) fed on a conventional diet either supplemented (+bacitracin) or not (control) with zinc bacitracin (100 ppm) and nurturing 5 or 9 pups.

		Diet			2-way ANOVA		
	Litter Size	Control	+Bacitracin	SE	Diet	LS	D x LS
N Intake (g/d)	**9 pups**	7.99	7.92	0.575	NS	NS	NS
	5 pups	7.85	8.02				
N Excretion							
Milk	**9 pups**	4.75	4.73	0.315	NS	NS	NS
	5 pups	4.56	3.89				
Faeces	**9 pups**	2.07	2.06	0.348	NS	NS	NS
	5 pups	2.43	2.18				
Urine	**9 pups**	3.04	3.13	0.880	NS	NS	NS
	5 pups	2.85	4.05				
N Balance (g/d)	**9 pups**	-1.87	-2.08	1.015	NS	NS	NS
	5 pups	-1.99	-2.00				

NS, *P*>0.05; SE, standard error; LS, litter size; D, diet.

Figure 1. Plasma enrichment (MPE, molar percent excess) of free phenylalanine (Average, n = 16) in plasma after 12, 15, 20, 25, 30 and 40 min of isotope infusion and in tissues after slaughtering, in rabbit does supplemented or not with bacitracin (100 mg/kg DM) and adjusted by cross-fostering at 5 or 9 pups.