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Research Paper

Responses of Plasma Acetate Metabolism to Hop (*Humulus lupulus* L.) in Sheep

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Abstract

An isotope dilution method using [1-^{13}C]sodium (Na) acetate was conducted to determine the effect of feeding hop (*Humulus lupulus* L.) residues on plasma acetate metabolism in six adult crossbred sheep. The sheep were fed 63 g/kg $BW^{0.75}$/d of either mixed hay (MH-diet) of orchardgrass (*Dactylis glomerata* L.) and reed canarygrass (*Phalaris arundinacea* L.) at a 60:40 ratio or MH-diet and hop-residues (Hop-diet) at 85:15 ratio with a crossover design for each of 3 week period. The isotope dilution method using single injection of [1-^{13}C]Na acetate was performed thrice; before feeding (BF), 2 h after feeding (2F) and 4 h after feeding (4F), on the 21st day of each dietary treatment. Plasma acetate concentration tended to increase (P= 0.06) and turnover rate was numerically higher (P= 0.16) for MH-diet than Hop-diet. Plasma glucose, NEFA, VFA and lactic acid concentrations were similar between dietary treatments. In both the diets, although plasma concentration of acetate did not change, turnover rate increased significantly (P= 0.02) 2F than BF. Hop-residues did not show any negative impacts on acetate metabolism as well as physiology of animals in the present experimental conditions, hence thereby it could be used as an alternative to MH-diet for rearing sheep.

Key words: Hop, [1-^{13}C]sodium acetate, stable isotope, acetate metabolism, sheep.

1. Introduction

Hop (*Humulus lupulus* L.) is one of the perennial climbing plants native to Europe, Asia and North America. Hop has been used in traditional medicine and as an ingredient in beer for many centuries and the breakthrough of hop chemistry came during the mid 20th century [1]. Hops are mostly grown in limited countries, located in northern hemisphere due to its photoperiodic sensitivity, and total amount produced in 1994 throughout the world was 121,000 tons [2]. Japan is also one of the hop producing countries of the world, produced about 415 tons of hop in 2006, of which about 98% was produced in northern Tohoku region [3]. The amount of hop-residues produced, is much greater than the amount used in beer production purpose. Despite the large quantities available for using as animal feed in hop producing countries, commercially has not been used for feeding ruminants because of lack of practical feeding trial using animals.

Hop is known to have two bitter compounds humulone and lupulone, flavonoids, phenolic compounds. Phenolics are antioxidants which influence the taste of food and improve the consumer's health [4]. Hop is mostly used in beer producing industries. However, the residues, which are composed of stem, leaves and unused flowers, are thrown or simply

disposed of. It is expected that these residues could be used as an animal feed facilitating the proper utilization of the residues as well as minimizing animal feed costs and preventing environmental pollution [5].

Acetate is the most important metabolite being involved in fatty acid and carbohydrate metabolism and the production of this acid largely depends upon the quality of feed, such as fiber contents [6, 7]. Energy substrate in food is the major determinant of blood acetate concentration, and blood acetate is a useful index of nutrition [8].

Yet, until now, to our knowledge, little information is available regarding the use of hop-residues as an animal feed. Since hop is rich in some chemical properties such as polyphenols, it was expected to be used as an antioxidant in ruminant feedstuffs. Therefore, the effect of hop on intermediate nutrients metabolism should be studied intensively. The present experiment was conducted to know the effects of hop-residues on the acetate metabolism in sheep. Moreover, the effect of time relating to feeding on acetate metabolism was also studied.

2. Materials and Methods

Animals, diets, and management

The handling of animals including surgery, cannulation and blood sampling was carried out according to the rules and regulations established by the Animal Care Committee of Iwate University. Six crossbred (Corriedale x Suffolk) adult shorn sheep of both sexes (*Ovis aries* L.), and 49.6±1.5 kg of body in weight (BW) were used. The animals were surgically prepared with a skin loop enclosing the left carotid artery under anesthesia at least three months before the start of the experiment. The sheep were housed in individual pens in a sheep barn during the adjustment period, first 2 weeks of the experiment. Two dietary treatments, one is mixed hay (MH-diet) of orchardgrass (*Dactylis glomerata* L.) and reed canarygrass (*Phalaris arundinacea* L.) at 60:40 ratio (7.5 MJ metabolizable energy (ME)/kg, and 94 g crude protein (CP)/kg air dry matter) and another one is that where 15% of MH-diet was replaced by air dried hop-residues (Hop-diet, *Humulus lupulus* L.) were used (the proximate components of mixed hay and hop-residues were shown in Table 1). In both dietary treatments the ME and CP intake were assumed to be maintained around isoenergetic and isonitrogenous at maintenance level [9]. The animals were fed 63g /kg $BW^{0.75}$/d once daily at 14:00 h, and usually ate everything within 2 h, although the manger was remained until the next morning. No preferential eating was observed between Hop-diet and MH-diet by the animals. Water was available *ad libitum*. The experiment was performed using a crossover design with two 3 week periods in which either Hop-diet or MH-diet was fed. Three sheep were fed the Hop-diet during the first period and then the MH-diet during the second period, but the other three were fed in the reverse order. On the 15th day, the animals were moved to metabolic cages in a controlled environmental chamber at a temperature of 23±1 °C, with 70% relative humidity and lighting from 08:00 h to 22:00 h. An isotope dilution method using [1-^{13}C]Na acetate and blood samples collection was conducted on the 21st day of each dietary treatment to determine plasma acetate metabolism. The sheep were weighed at starting of the experiment, on the 15th day and after finishing of each dietary treatment.

Two catheters, one for isotope infusion and another for blood sampling, were inserted into the right jugular vein and left carotid artery, respectively on the morning of each determination of isotope dilution method. Catheters were filled with sterile solution of trisodium citrate (0.13 mol/L). Blood sampling were performed without any noticeable stress to the sheep.

Table 1. Proximate composition and polyphenol contents of hop-residues (*Humulus lupulus* L.), and mixed hay (MH) of orchardgrass (*Dactylis glomerata* L.) and reed canarygrass (*Phalaris arundinacea* L.) (60:40).

	Hop	MH
Dry matter (%)	84.5	91.9
Crude protein (% DM)	17.6	10.2
Crude fiber (% DM)	13.2	28.8
Ether extract (% DM)	9.7	3.0
Crude ash (% DM)	29.3	11.7
Nitrogen-free extract (% DM)	30.2	46.3
Polyphenol (mg/g DM)	2.0	ND*

*ND= not detected; DM=dry matter

Experimental procedures

Collection of rumen fluid

On the 20th day of each dietary treatment, rumen fluid (50 ml) was taken at 2 h after feeding (2F) via a stomach tube inserted orally into the rumen during collection. The liquid fraction of the ruminal fluid was then separated by centrifuging at 5,000 x *g* (RS-18 IV, Tomy, Tokyo, Japan) for 10 min. An aliquot was stored at -30 °C until the analyses.

Isotope dilution method of [1-^{13}C]Na acetate

At 12:00 h on the day of isotope dilution

method, 2.2 mmol of [1-^{13}C]Na acetate (1-^{13}C, 99% CLM-156-10, Cambridge Isotope Laboratories, Inc. MA, USA) dissolved in 10 ml of saline solution (0.15 mol sodium chloride/L) was injected through the jugular infusion catheter as a single injection 15 min before feeding (BF). The same isotope with the same volume was also injected 2F and 4 h after feeding (4F), respectively. The background blood samples (8 ml) were collected from the sampling catheter immediate before, and at 2, 4, 6, 8 and 10 min after each injection of the isotope for the determination of plasma acetate pool size and turnover rate from the plasma isotopic enrichment of [1-^{13}C]Na acetate. Blood samples were transferred into centrifuge tubes containing one drop of heparin solution (4,000 U/ml) and were chilled until centrifugation.

Chemical analyses

Analysis of proximate components of the experimental diets were performed according to AOAC [10] as described before [11] and the total polyphenol contents of hop was determined according to Folin-Denis method [12] as described in our previous paper [13]. The NH_3-nitrogen (N) content of the rumen fluid was determined using colorimetric method [14]. Rumen total volatile fatty acid (VFA) concentrations were determined after titrating the steam distillate of rumen fluid with 0.1 N NaOH. The titrated distillate was dried and then the molar percentages of individual VFA were determined using a gas chromatography (GC) (HP5890A, Hewlett Packard, Avondale, USA).

Blood samples were centrifuged at 10,000 x *g* for 10 min at 2 °C (RS-18 IV, Tomy, Tokyo, Japan), and the plasma samples were then stored at -30 °C awaiting further analysis. Plasma [1-^{13}C]acetate enrichment and VFA concentrations were determined using selected ion monitoring system with gas chromatography mass spectrometry (GC/MS) (QP-2010, Shimadzu, Kyoto, Japan) according to the method described by Moreau et al. [15]. The following ions were determined: for the [1-^{13}C]acetate enrichment m/z 117 and 118, and for the acetic, propionic, butyric, valeric and lactic acids m/z 117, m/z 131, m/z 145, m/z 159 and m/z 261, respectively. The 0.5 mmol /L 2-ethylbutyric acid and 0.5 mmol/L 4-methylvaleric acid were used as internal standards. Concentration of plasma glucose was enzymatically determined by the method of Huggett and Nixon [16]. Concentrations of plasma nonesterified fatty acids (NEFA) were also enzymatically determined with a kit (NEFA C test, Wako Pure Chemicals, Osaka, Japan).

Calculations

Mean values with standard errors are given. Plasma acetate pool size and turnover rate were calculated according to a single-pool analysis [17]. The sampled pool represents the blood which is in rapid equilibrium with the interstitial fluid [18].

$$\text{Pool size} = \text{Quantity of isotope added}/IE_0$$

Where, IE_0 is the isotopic enrichment at time zero (0).

$$\text{Half- life} = \ln 2/k_e$$

Where, k_e is the rate constant for elimination.

$$\text{Turnover rate} = \text{Pool size}/\ k_e$$

Statistical analysis

All data were analyzed with the MIXED procedure of the SAS [19]. The least-square means statement was used to test the effects of period, diet, feeding time and interaction of diet and time. The random effect was sheep. Results were considered significant at the $P<0.05$ level, and a tendency was defined as $0.05< P<0.10$. The repeated statement and the Tukey-Kramer adjustment were used for the time course of changes and the significance were $P<0.05$ and $P<0.10$, respectively.

3. Results and Discussion

Rumen parameters

Rumen NH_3-N concentration tended to be higher for Hop-diet ($P= 0.08$) than MH-diet. No significant variations in the total as well as individual rumen VFA concentrations were found in the present study (Table 2). Although the diets were assumed to be isonitrogenous and isoenergetic, the CP contents of PL-diet were numerically higher than the MH-diet. The higher rumen NH_3-N and slightly higher rumen VFA for Hop-diet might be due to the higher CP and lower CF in hop than mixed hay (Table 1). As Fujita et al. reported positive influence of dietary CP contents to rumen VFA concentration in goats [20], and to both of the rumen NH_3-N and VFA in cattle [21]. Total rumen VFA and acetate concentrations of the present study were a little higher, and rumen butyrate and propionate concentrations were comparable with the findings in goats fed alfalfa hay, soybean meal and cornstarch by Fujita et al. [20]. The difference in total VFA and acetate concentrations might be due to the type of diets differing in fiber content. For instance, Sutton et al. [6] stated that the marked changes in the molar proportions of the concentrations of rumen VFA could be induced in response to a wide variety of dietary manipulations.

Table 2. Effect of hop (*Humulus lupulus* L.) on ruminal characteristics at 2 h after feeding in sheep.

Rumen parameters	Treatment*		SEM	P-value
	Hop-diet	MH-diet		
No. of sheep	6	6		
Ammonia nitrogen (mg/dL)	24.6	18.2	1.6	0.08
VFA conc. (mmol/L):				
Total	79.3	75.9	7.1	0.35
Acetate	62.2	61.3	3.8	0.39
Propionate	11.0	9.6	0.8	0.36
Butyrate	3.7	3.1	0.3	0.36
Iso-bytyrate	0.8	0.6	0.1	0.27
Valerate	0.6	0.5	0.1	0.26
Iso-valerate	1.0	0.8	0.1	0.29

*MH-diet= orchardgrass and reed canarygrass (60:40) hay; Hop-diet= MH-diet and hop (*Humulus lupulus* L.)(85:15); SEM= standard errors of the means; VFA= volatile fatty acids.

Plasma acetate turnover rate

The plasma acetate concentration was tended to lower (P= 0.06) for Hop-diet than MH-diet (Table 3) which might be due to the lower crude fiber contents of the hop than the mixed hay because; the acetate production is influenced by the fiber contents of the diet. For example Sutton et al. [6] infused isotopically labeled acetate, propionate and butyrate into the rumen of dairy cows fed normal and low roughage diets and found that acetate production was slightly lower for low roughage diet.

Table 3. Effect of hop (*Humulus lupulus* L.) on plasma VFA, lactate, NEFA and glucose concentrations in sheep.

	Treatment*						SEM	P-value		
	Hop-diet			MH-diet				Diet	Time	Diet x Time
	#BF	2F	4F	BF	2F	4F				
No. of sheep	6	6	6	6	6	6				
Total VFA (µmol/L)	661	671	730	717	716	831	35	0.13	0.17	0.85
Acetate (µmol/L)	570	577	640	650	631	749	29	0.06	0.16	0.86
Propionate (µmol/L)	31	33	26	14	28	18	3.4	0.21	0.58	0.82
Butyrate (µmol/L)	13	18	19	11	14	16	1.4	0.13	0.05	0.90
Iso-bytyrate (µmol/L)	21	22	19	22	22	25	0.8	0.24	0.92	0.28
Valerate (µmol/L)	6	4	6	5	5	6	0.3	0.74	0.67	0.89
Iso-valerate (µmol/L)	19	16	20	15	16	18	0.8	0.50	0.71	0.76
Lactate (mmol/L)	0.31	0.23	0.28	0.30	0.25	0.34	0.02	0.38	0.08	0.51
NEFA conc. (mEq/L)	0.25	0.25	0.18	0.20	0.25	0.20	0.01	0.70	0.13	0.45
Glucose conc. (mmol/L)	2.71	2.21	2.38	2.66	1.94	2.37	0.07	0.73	<0.0001	0.95

*MH-diet= orchardgrass and reed canarygrass (60:40) hay; Hop-diet= MH-diet and hop (*Humulus lupulus* L.) (85:15); BF= before feeding; 2F= 2 hours after feeding; 4F= 4 hours after feeding; SEM= standard errors of the means; VFA= volatile fatty acids; NEFA= non-esterified fatty acids.

The butyrate concentrations increased (P= 0.05) gradually after feeding irrespective of diets in the present experiment. The butyrate concentration was slightly higher (P= 0.13) for Hop-diet than MH-diet. In both dietary treatments lactate concentrations tended (P= 0.08) to change, either increase or decrease, in relation to feeding. The total plasma VFA concentration was numerically lower (P= 0.13) for Hop-diet than the MH-diet.

Plasma NEFA concentration was slightly greater (P= 0.70) for Hop-diet than MH-diet. In a previous report it was stated that plasma NEFA is the best indicator of body lipid loss [22]. However, in the present experiment the difference might be due to the compositional variation as the Hop-diet was higher in crude fat contents than the MH-diet. In both diets the numerical value of concentrations of NEFA were higher 4F, which might be due to the fact that the energy requirements was fulfilled with acetate as the plasma acetate concentration were numerically higher 4F in both diets. The numerical value of plasma NEFA in the present experiment was comparable to our previous findings in sheep fed mixed hay and a herb, plantain (*Plantago lanceolata* L.) [23].

Plasma glucose concentration was comparable between diets. Although plasma propionate, the most important single precursor of glucose was numerically higher for Hop-diet than MH-diet. Propionate concentration was numerically lower in Hop-diet which indicates concentrate type of fermentation as another herb plantain [24]. In a previous study [25], it was reported that infusion of propionate into rumen failed to influence glucose percentage derived from propionate in goats. The decrease of plasma glucose concentration 2F might be due to the low rate of digestibility and absorption.

The average pooled values of biological half-life of acetate were about 2 min in both dietary treatments which were very similar to the previous findings in sheep (26) (Table 4). The plasma acetate pool size and turnover rate were numerically lower (P= 0.12 and P= 0.16, respectively) for Hop-diet than MH-diet. In case of Hop-diet, the pool size was numerically higher after feeding and the value of 2F was higher (P= 0.03) than that of 4F. In case of MH-diet, the value also increased after feeding, but unlike Hop-diet, the value was numerically higher 4F than that of 2F. The inconsistency in post feeding acetate pool size between diets reveals that the Hop-diet might contain more soluble carbohydrate or iono-

phore antibiotic like salinomycin than MH-diet [27, 28]. Plasma acetate turnover rate was higher (P= 0.02) 2F and numerically higher 4F than that of BF in both the dietary treatments. The numerical value of acetate turnover rate of the present study was higher than found in a previous study by Sunagawa et al. [29]. They determined acetate metabolism using [1, 2-^{14}C]Na acetate isotope dilution technique in sheep fed alfalfa hay cube and concentrate mixture twice daily. The inconsistency in acetate turnover rate might be due to several factors; for example, they used radioactive isotope and infused continuously to determine acetate turnover rate during 6 to 8 h after feeding. Moreover, the difference of feed, frequency of feeding might also partially be the contributing factors of the above inconsistency. In a previous study, Lee and Williams [30] determined acetate turnover rate 2F-4F in cattle and stated that the rate of acetate utilization doesn't depend on concentration. Our findings in the current study very much agree with them. However, the value found by Lee and Williams [30] was lower than the present findings which might be due to the difference of species and feed.

Table 4. Effect of hop (*Humulus lupulus* L.) on plasma acetate pool size and turnover rate in sheep.

	Treatment*						SEM	P-value		
	Hop-diet			MH-diet				Diet	Time	Diet x Time
	BF	2F	4F	BF	2F	4F				
No. of sheep	6	6	6	6	6	6				
Acetate pool size (mmol/kg$^{0.75}$)	0.42	0.58	0.50	0.47	0.53	0.65	0.03	0.12	0.03	0.79
Acetate turnover rate (mmol/kg$^{0.75}$/min)	0.15	0.20	0.17	0.17	0.22	0.20	0.02	0.16	0.02	0.92
Half-life of acetate (min)	2.0	1.8	1.9	2.1	2.0	2.1	0.1	0.16	0.30	0.95

*MH-diet=orchardgrass and reed canarygrass (60:40) hay; Hop-diet=MH-diet and hop (*Humulus lupulus* L.) (85:15); BF=before feeding; 2F=2 hours after feeding; 4F=4 hours after feeding; SEM=standard errors of the means.

4. Conclusions

It was expected that plasma acetate metabolism would increase due to the bioactive components, polyphenol content in hop. However, no positive impact was found in the present experimental diet containing 15% of the dietary dry matter as hop-residues in sheep. Therefore, it could be concluded that the hop-residues could be considered as an alternative to MH-diet for rearing sheep. It is suggested that further experiment should be performed to study other nutritional parameters such as plasma glucose and amino acids kinetics in ruminants increasing the amount of hop-residues.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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