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Identification of Glucogenic Amino Acids Content in *Gliricidia maculata* as an Alternative Energy Source for High-Yielding Periparturient Dairy Cows

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ABSTRACT

Gliricidia maculata which has long been used as a forage for animal feed may contain a variety of glucogenic amino acids variants. The objective of this study was to identify the glucogenic amino acids content in *Gliricidia maculata* as an alternative source of energy for high-yielding periparturient dairy cows. The samples were the edible portion of plants, harvested randomly at the optimal cutting age (80 days), from the feed plant collection garden of The Faculty of Animal Science, Universitas Gadjah Mada. Lyophilization was carried out by drying the samples at 55°C for 3 x 24 hours continouesly in a Sanyo Drying Oven MOV-112. Pulverization was done by the Foss Tecator Cyclotec™ 1093 Sample Mill with 300 mesh (1 mm screen). The hydrolysis of amino acid was carried out using HCl solution while amino acid derivatization used O-phtalaldehyde (OPA) solution. Separation, determination, and quantification of amino acid were carried out by an analytical method in gradient elution using the Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC Systems with Rapid Separation Fluorescence Detector. Result showed there were at least fourteen kinds of amino acids identified from the samples, i.e.: aspartic acid, glutamic acid, serine, histidine, glycine, arginine, alanine, tyrosin, methionine, valine, phenylalanine, isoleucine, leucine and lysine. *Gliricidia maculata* contains 1349 ppm glucogenic amino acids, 412.7 ppm ketogenic amino acids and 444.7 ppm glucogenic and ketogenic amino acids. Moreover, there were three types of glucogenic amino acids with the highest concentration were serine (288.7 ppm), glutamic acid (245.5 ppm) and phenylalanine (197.1 ppm) respectively. Glucogenic amino acids can be used as an energy source for dairy cows through gluconeogenesis. This study suggests that *Gliricidia maculata* may use to supply the precursors of energy for high-yielding periparturient dairy cows to prevent ketosis.

Keywords: *Gliricidia maculata*, Glucogenic amino acids, Periparturient dairy cows

Introduction

The transition from pregnancy to lactation is crucial for the profitability of the dairy cow. Understanding important aspects of the biology of the transition cow helps guide feeding and management decisions (Grummer, 1995; James, 1999). Dairy cattle are at increased risk for many disease and disorders during a transition period or a periparturient period (Leslie *et al*., 2003). The transition period was defined as the period from 3 weeks pre-calving until 3 weeks post-calving. The period is characterized by marked changes in the endocrine status of animal and a reduction in feed intake when nutrient demand for the developing fetus and the impending lactogenesis are increasing (Grummer, 1995). At this time, there is incising milk production but a lag in feed intake, so create a negative energy balance – NEB (Leslie *et al*., 2003). Energy balance is the most critical nutritional factors impacting on animal health, lactation and reproductive performance (Van Saun, 2002; 2008). Approximately 75% of disease in adult dairy cows typically happens in the first month after calving (LeBlanc *et al*., 2006) with the highest incidence of total disease (mastitis, ketosis, digestive disorders, and lameness) taking place within the first 10 days post-calving (Goff and Horst, 1997; Ingvartsen *et al*., 2003). Disease prevention and maintenance of adequate production levels are the greatest challenges in high-yielding dairy herds during transition from the late dry period to the first weeks of lactation (LeBlanc *et al*., 2006).

Dairy cow ketosis is a common nutrition metabolic disease occurring in the initial stages of lactation in high-yielding dairy cows, caused by carbohydrate and fat metabolism disorder or nutritive deficiency that leads to an increased susceptibility to infectious and metabolic diseases,

as well as fertility disorders (Baird, 1982; Erb and Grohn, 1988; Grummer, 1993; Duffield *et al*., 2009; McArt *et al*., 2013).

Ketosis may occur in the primary, secondary and alimentary form. The primary cause of ketosis is a NEB postpartum, which results in lipolysis of fat (triglycerides) from adipose tissue and degradation of body proteins, thus large quantities of non-esterified fatty acids (NEFA) in the blood. In the liver, after the conversion into acetyl-CoA, NEFA may be fully oxidized or transformed into ketone bodies (acetoacetic acid, β-hydroxybutyric acid (BHBA) and acetone) during the process of ketogenesis (Duffield, 2000; Xu and Wang, 2008; Duffield *et al*., 2009; González *et al*., 2011; Marczuk *et al*., 2018).

Pathogenesis of ketosis combines several different interrelated metabolic processes such as glycolysis, lipolysis, proteolysis, gluconeogenesis, as well as metabolism of amino acids and fatty acids. Milk production in the initial lactation period increases the need for glucose. Glucose is essential for the proper functioning of central nervous system (CNS) cells (glial cells or neuroglia), red blood cells and the synthesis of milk components. In the process of glycolysis, glucose is degraded to pyruvate. Depending on the conditions, pyruvate may be converted into acetyl-CoA and could enter the Krebs cycle, or may be converted into lactate or alanine – which are the precursors of glucose in the process of gluconeogenesis. Glycolysis results in the decomposition of glucose and formation of energy in the form of adenosine triphosphate (ATP). During the esterification process, NEFA may also be converted again into triglycerides, for which the excessive amounts are accumulated in hepatocytes (Marczuk *et al*., 2018). The accumulation of triglycerides in hepatocytes may causing hepatic steatosis (fatty liver disease), thus reducing liver function. Furthermore, ketosis in dairy cows can decrease milk production and affect economic performance.

Gluconeogenesis occurs in parallel to the lipolysis processes, i.e., the formation of glucose from fatty acids of rumen and non-carbohydrate compounds as well. After feeding and in the period of high energy consumption, gluconeogenesis using short chain fatty acids (propionate, valerate, and isobutyrate) of rumen is the highest. A decreased feed intake observed in the course of ketosis involves the use of non-carbohydrate compounds for gluconeogenesis. In this case, substrates for gluconeogenesis are lactic acid, glycerol, and amino acids such as glutamate, aspartate, glycine, histidine, proline, glutamine, and valine (Aschenbach *et al*., 2000).

Leaf protein sources obtained in leaf vegetables, legume trees, browse plants, fodder trees and shrubs as feed resources to all classes of livestock offer tremendous potentials and are receiving increasing attention (Aye, 2007; Asaolu *et al*., 2011). *Gliricidia sp*. is a tropical tree legume that has been widely employed as plantation shade, green manure, living fence posts, firewood, and livestock fodder. Available data indicate that *Gliricidia sp*. is rich in protein (20 – 30% of the dry matter), a crude fibre content of only about 15%, and in vitro dry matter digestibility of 60 – 65% (Simons and Stewart, 1994). High levels of protein in *Gliricidia maculata* may contain lots of amino acids, especially glucogenic amino acids. The aim of this research was to identify the glucogenic amino acids content in *Gliricidia maculata*, as an alternative energy source for high-yielding periparturient dairy cows.

Materials and Methods

The study was started by sampling of *Gliricidia maculata* from the feed plant collection garden of The Faculty of Animal Science, Universitas Gadjah Mada feed plant collection. The samples were harvested randomly at the optimal cutting age (80 days), in June 2019 in the afternoon (15:00 to 16:00 h), from the edible portions of plants, include: leaves, twigs and small stems. Immediately after harvesting, the samples were packaged into a paper bag then weighed by an Explorer® Semi-Micro Ohaus E12140 Analytical Balances for getting a fresh weight (as fed). Evaporation of water molecules (lyophilization) was carried out at 55°C for 3 x 24 hours continouesly using Sanyo Drying Oven MOV-112. Reduction of particle size of simplicia (pulverization) was carried out by Foss Tecator Cyclotec™ 1093 Sample Mill (300 mesh, 1 mm screen). The hydrolysis of amino acid used a 6N HCl solution at 110ºC for 24 hours while the amino acid derivatization used an ortho phtalaldehyde (OPA) solution. Separation, determination, and quantification of amino acid were carried out by an analytical method in gradient elution using the Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC Systems with Rapid Separation Fluorescence Detector. The column of Hypersil GOLD (5µm diameter and 250 x 4.6 mm length) was used by this instrument. The mobile phase A was the solution consisting of methanol (CH_3OH), 50mM sodium acetate (CH_3COONa) and tetrahydrofuran (THF) in a composition of 2:96:2 with a pH of 6.8 and the mobile phase B was 65% CH_3OH. The gradient program starts with a composition of 100% A: 0% B for 2 minutes then was changed to 0% A: 100% B for 35 minutes (it held for 40 minutes before returning to the initial condition). The specified flow rate was 1.5ml/minute. A standards solution was made by dissolving successively: 50 ppm, 100 ppm, dan 250 ppm of amino acids standard added with the OPA solution up to 300 mL and stirred for 5 minutes at room temperature. This study used 14 of amino acid standards available at Organic Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, i.e.: aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), arginine (Arg), alanine (Ala), tyrosine (Tyr), methionine (Met), valine (Val), phenylalanine

(Phe), isoleucine (Ile), leucine (Leu) and lysine (Lys). A linear regression curves (y) were obtained by injected 20µL of each standard solution to the UHPLC instrument. Determination of amino acid concentration (x) was based on the area under the curve formed in the chromatogram, resulting from pre-column amino acid derivatization by an OPA solution (Figure 1) and the linear regression curves (y). The final step in this research was classify amino acids into three groups, i.e.: (1) glucogenic amino acids, (2) ketogenic amino acids, (3) glucogenic and ketogenic amino acids, then calculated the total content for each group.

Results and Discussion

In healthy cows, with maintained appetite, the main glucose precursors include propionate (60 – 74%), L-lactate (16 – 26%), valerate and isobutyrate (5 – 6%), glycerol (0.5 – 3%) and small amounts of glucogenic amino acids (Glu, Asn, Gly, His, Pro, Gln, and Val) (Aschenbach *et al*., 2000; Larsen and Kristensen, 2013; Sun *et al*., 2014). The absence or decreased appetite in cows suffering from ketosis causes deficiency of the mentioned substrates, and therefore there is an activation of gluconeogenesis using amino acids, which was confirmed by other authors (Li *et al*., 2014; Sun *et al*., 2014). The concentration of glucogenic amino acids (Ala, Asp, His, Met, Pro, and Ser) in blood serum of dairy cows with primary ketosis were decreased (Marczuk *et al*., 2018). Low concentrations of these amino acids may occur due to their intensive use in the process of gluconeogenesis (Shibano and Kawamura, 2006; Maeda *et al*., 2012). According to the literature data, the intermediates, i.e. pyruvate and keto acids (h-ketoglutarate, succinyl CoA, fumarate, and oxaloacetate) are formed from glucogenic amino acids during metabolic processes. They participate in the metabolic reactions of the Krebs cycle, creating CO_2, H_2O, and energy in the form of ATP. In the case of high demand for glucose, oxaloacetate falls out of the Krebs cycle and is used for glucose synthesis (Xu *et al*., 2008).

In dairy cows with ketosis, due to low concentration of glucogenic amino acids, gluconeogenesis is inhibited. Simultaneously, the lack of glucogenic amino acids leads to the disruption of the Krebs cycle reaction (interruption of the TCA cycle). It is evidenced by the decreased concentration of citrate – intermediate metabolite of the Krebs cycle, among dairy cows with ketosis (Zhang *et al*., 2013). Low concentration of essential amino acids (Met) demonstrated in cows with ketosis could result from their low content in the feed ration and/or low intake of feed. Met is a methyl donor for the synthesis of phospholipids – essential components of very low-density lipoprotein (VLDL) which is responsible for the removal of triglycerides from the liver. In cows with ketosis, we demonstrated a low concentration of low density lipoprotein (LDL) and VLDL, which may indicate the disruption of their synthesis caused by, among others, Met deficiency (Sun *et al*., 2014).

The secondary ketosis in the course of left displacement of abomasum (LDA) (Hamana *et al*., 2010) and fatty liver syndrome was demonstrated by other authors (Hidiroglou and Veira, 1982; Pechova *et al*., 2000; Shibano and Kawamura, 2006; Imhasly *et al*., 2014) concurrently with low concentrations of glucogenic amino acids (asparagine, glutamine, glycine, methionine) and essential amino acids (arginine, phenylalanine, threonine) and high concentration of ketogenic amino acids (leucine, lysine). The studies

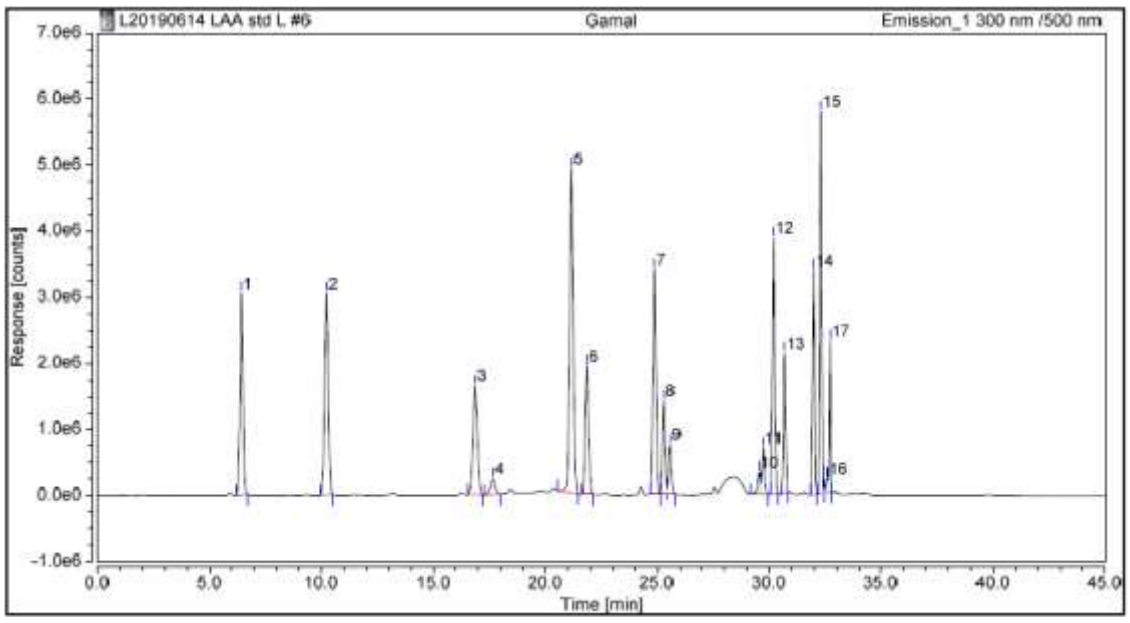

Figure 1. Chromatogram of amino acid resulting from pre-column derivatization by an OPA solution.

Table 1. Concentration of amino acids in *Gliricidia maculata* (ppm)

Amino acid (AA)	Retention time	Area under the curve	Linear function of standard curve	Concentration (ppm)
Glucogenic AA				
Asp	6.43	460867.67	y = 2608.8x – 9349.4	180.2
Glu	10.22	567812.48	y = 2331.2x – 4401.3	245.5
Ser	16.86	371313.71	y = 1294.6x – 2490.8	288.7
His	17.65	55487.58	y = 773.47x – 5340.9	78.6
Gly	21.16	915227.84	y = 7388.9x – 20727	126.7
Arg	21.85	338815.87	y = 3195.3x – 3895.5	107.3
Ala	24.87	527261.18	y = 4110x – 1583.8	128.7
Met	29.73	87544.47	y = 2329.5x + 1742.7	36.8
Val	30.19	469657.56	y = 2975.3x + 4071.3	156.5
Total				1349
Ketogenic AA				
Leu	32.30	494871.83	y = 2290.7x + 2314.2	219.1
Lys	32.72	129322.83	y = 688.93x – 4076	193.6
Total				412.7
Glucogenic and ketogenic AA				
Tyr	25.29	184206.35	y = 1594.5x – 2352.2	117.0
Phe	30.68	234534.54	y = 1192.3x – 452.77	197.1
Ile	31.98	325526.67	y = 2453x + 5165.4	130.6
Total				444.7

conducted on dairy cows with the primary ketosis also demonstrated some changes in the concentration of amino acid metabolism (2-piperidinecarboxylic acid, 3-hydroxyisovaleric acid, 4-aminobutyric acid), which indicates their active participation in the ketosis pathogenesis (Zhang *et al*., 2013; Sun *et al*., 2014).

Perhaps, the prevention of this disease is possible by appropriate feed ration balancing in terms of amino acid content. Glucogenic amino acids can be used as an energy source for dairy cows through gluconeogenesis. If the body is not optimally utilizing the glucogenic amino acids, ketogenic amino acids can be used as a second opinion for supplying the precursors of energy during the periparturient period to prevent ketosis. According to this study, *Gliricidia maculata* almost contains all types of amino acids (glucogenic amino acids, ketogenic amino acids, and also glucogenic and ketogenic amino acids), with the total content of each group were 1349; 412.7; and 444.7 ppm respectively. The three types of glucogenic amino acids with the highest concentration were serine (288.7 ppm), glutamic acid (245.5 ppm) and phenylalanine (197.1 ppm) respectively (Table 1). This is a fairly large concentration when compared to the amino acid levels in other legumes (Astuti and Widyobroto, 2019). *Gliricidia sp*. is rich in protein (20 – 30% of the dry matter), a crude fibre content of only about 15%, and highly digestible (in vitro dry matter digestibility of 60 – 65%) (Simons and Stewart, 1994). Thus, based on the results of the previous research and this study, *Gliricidia maculata* can be used as an alternative source of energy for high-yielding periparturient dairy cows.

Conclusion

Gliricidia maculata contains 1349 ppm glucogenic amino acids, 412.7 ppm ketogenic amino acids and 444.7 ppm glucogenic and ketogenic amino acids. The three types of glucogenic amino acids with the highest concentration were serine (288.7 ppm), glutamic acid (245.5 ppm) and phenylalanine (197.1 ppm) respectively. This study suggests that *Gliricidia maculata* may use to supply the precursors of energy for high-yielding periparturient dairy cows to prevent ketosis.

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