A THESIS FOR THE DEGREE OF MASTER

# Correlation between the microbiome and blood lactate concentration in canines with insulin-dependent diabetes mellitus

## 인슐린 의존성 당뇨 환견의 마이크로바이옴과 혈중 젖산 농도의 상관관계

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수의학과 임상수의학 (수의내과학) 전공
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이 논문을 수의학 석사 학위논문으로 제출함

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# Abstract

## Correlation between the microbiome and blood lactate concentration in canines with insulin-dependent diabetes mellitus

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The correlation between the intestinal microbiome and endocrine disorders has recently been drawing attention as an important key for determining their pathology and clinical assessment. Recent evidence suggests a correlation between dysbiosis and the pathogenesis of insulin-dependent diabetes mellitus (IDDM). However, these studies remain controversial, and further studies are needed to define a gut microbiome imbalance in IDDM and identify the causative factors. Poor diabetes management can increase the blood lactic acid in dogs as well as humans. Previous studies have reported a correlation between intestinal dysbiosis and lactate concentration. In this study, the microbiome of dogs with IDDM were evaluated with respect to blood lactate. All subjects were categorized into diabetic and healthy (non-

diabetic) dogs. Dogs with naturally occurring IDDM (n = 11) and healthy dogs (n = 6) were enrolled and also dogs with IDDM were divided into two groups: those with normal blood lactate levels (N-DM, n=5) and those with levels above the normal range (L-DM, n=6). All group's diabetes mellitus control indicators were measured. Using RT-qPCR, lactate producing bacteria and dysbiosis associated bacteria are measured by relative expression with universal mRNA. Expression levels of the lactate-producing bacteria *Lactobacillus* spp., *Enterococcus* spp., and *Bifidobacterium* spp., were confirmed in IDDM patients comparing with healthy dogs. In diabetes mellitus dogs, relative mRNA expression of *Enterococcus* spp. and *Bifidobacterium* spp. increased 6.23, 3.90-fold respectively compared to healthy dogs. When blood lactate concentration increased in diabetes dogs, relative mRNA expression of *Bifidobacterium* spp. increased 3.90-fold compared to diabetes dogs with normal blood lactate levels. In conclusion, blood lactate levels influence the gut microbiome in dogs with IDDM. It is noteworthy that these findings are related to previously studied intestinal microbial flora. And the intestinal flora of diabetic patients changed according to their blood lactate levels, which means that the blood lactate concentration could be a major indicator of intestinal dysbiosis in diabetic patients. Our study will help understand the gut microbiota in the context of diabetes in human and veterinary medicine.

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**Keywords:** canine, insulin-dependent diabetes mellitus, microbiota, lactate, dysbiosis

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# 1. Introduction

Intestinal dysbiosis, an imbalance of the microbiome, is associated with metabolic disorders, obesity, insulin resistance, type 2 diabetes, inflammatory bowel disease, and impaired immune function [1]. Recent research on the fecal microbiome has attracted the attention of scientists and healthcare workers worldwide, focusing on the interaction of diabetes with the gut microbiome [2].

Insulin-dependent diabetes mellitus (IDDM) or type 1 diabetes (T1D), is the most common type of diabetes mellitus in dogs [3]. Persistent diabetes induces the loss of beta cells; hypoinsulinemia, which results in an insufficient supply of circulating glucose to most cells; and accelerated hepatic gluconeogenesis and glycogenolysis [4]. Recent evidence suggests a correlation between dysbiosis and the pathogenesis of IDDM. However, these studies remain controversial, and further studies are needed to define a gut microbiome imbalance in IDDM and identify the causative factors [2, 5].

Patients with IDDM, especially if poorly controlled, can store excess hepatic glycogen and exhibit increased plasma lactate levels [6]. Elevated blood lactate levels can lead to emergency complications in human IDDM patients. Previous studies have reported a correlation between intestinal dysbiosis and lactate concentration. When intestinal dysbiosis occur, blood lactate concentration of the patient increase [7]. However, there has been no study focusing on the underlying mechanism between increased blood lactate concentrations and the fecal microbiome in patients with IDDM.

In this study, we assessed the microbial communities in non-diabetic and diabetic dogs and hypothesized that blood lactate concentrations in diabetic dogs are a major factor influencing the gut microbiota.

# 2. Materials and Methods

### *2.1. Animals*

All subjects were categorized into diabetic and healthy (non-diabetic) dogs. Dogs who had any diseases which could affect their blood lactate concentrations were not included in this study. The normal circulating lactic acid level in dogs is ≤2.5 mmol/L [8]. Dogs with diabetes were divided into two groups according to their blood lactate concentration, within the normal range and above the normal range, according to the criteria.

### *2.2. Sample collection*

Dogs with naturally occurring IDDM (n = 11) and healthy dogs (n = 6) were enrolled from the hospital population at the Veterinary Medical Teaching Hospital of Seoul National University. Also, dogs with IDDM were divided into two groups: those with normal blood lactate levels (N-DM, n=5) and those with levels above the normal range (L-DM, n=6). Dogs with DM were diagnosed based on urinalysis and clinical signs. All dogs participated in this study after informed consent was obtained from their guardians. All protocols were previously approved by Seoul National University Institutional Animal Care and Use Committee and adhered to the guidelines (SNU-200912-3).

### *2.3. Sample analysis*

Blood was collected from both groups of dogs in a fasting state by routine venipuncture using the jugular or cephalic veins. Glucose, ketone (isens, caresensdual, Seoul, Korea), hemoglobin A1c (Hb1ac; Biattic, Aniscan, Gyeonggi-do, Korea), and lactate (nova biomedical, Stat Strip xpress lactate, como, Italia) were measured in fresh whole blood, while fructosamine (IDEXX, Catalyst one, NY, US) was measured in serum. Serum was separated quickly within 15 min of collection. A sample for blood gas analysis (isens, ismart, Seoul, Korea) was collected with a heparin-coated syringe. All dogs were fasted for at least 12 h before samples were obtained. Free catch urine was used. The presence of urine glucose was confirmed using urine test strips (Roche, Combur-Test®, Basel, and Swiss). The majority (n = 8) of dogs with IDDM were fed a prescription diet for diabetes and three dogs with IDDM were fed commercial maintenance rations. All control dogs were fed commercial maintenance rations. No dogs had a history of antibiotic administration for at least six months prior to sample collection. Fecal samples were also free catch and stored at -80 °C until all samples were collected.

### *2.4. RNA extraction, cDNA synthesis, and RT-qPCR*

Fecal RNA was extracted using an RNeasy Power Microbiome Kit following the manufacturer's instruction (Qiagen, Hilden, Germany). cDNA was synthesized by isolating sample RNA using Cell Script cDNA MasterMix (Cell-Safe) according to the manufacturer's instructions. A total of 10 μL of cDNA was synthesized and

diluted with DEPC. In this experiment, a total of seven bacteria were targeted and quantified using RT-qPCR (StepOne™ Real-Time PCR System, Thermofisher, Massachusetts, US). The target bacteria and primer sequences are shown in Table 1. For comparison, universal cDNA was measured together.

### *2.5. Statistical analysis*

All experiments were performed in triplicate and repeated three times. GraphPad prism (version 6.01) software (GraphPad, Inc., La Jolla, CA, USA) was used for statistical analysis. Comparisons of more than two groups were performed using one-way analysis of variance followed by Bonferroni's multiple comparison test. The results are presented as the means ± standard deviations. P-values $<0.05$ were considered statistically significant.

# 3. Results

### *3.1. Characteristic participants*

Signalment is described in Table 1. Healthy dogs had no specific abnormalities. Dogs with IDDM who normal lactate levels did not have any diseases that affected their blood lactate concentration such as urolithiasis, tracheal collapse, chronic valvular heart disease, or mast cell tumor resection history. Two dogs with IDDM who had increased lactate levels had hyperadrenocorticism, while the others had no diseases. Blood lactate concentration differences were evaluated between Diabetic and healthy dogs. Except for two dogs, dogs with IDDM had higher than normal blood glucose levels. The blood glucose of healthy dogs was within the normal range. Diabetic dogs also exhibited higher lactate concentrations. Diabetic dogs were further divided into high and normal lactate groups. Diabetes mellitus control indicators (fasting glucose, ketone, Hb1ac, and fructosamine) were measured (Figure 1). The DM groups had higher lactate, blood glucose, ketone, Hb1ac, and fructosamine levels than the healthy group. The L-DM group had significantly higher ketone levels than in the N-DM group. The DM groups had a higher blood pH compared to the healthy group, but there was no significant difference between the DM groups (Figure 2). There is no significant difference in pCO2 and pO2 between the DM and healthy groups. Bicarbonate was lower in the DM groups compared to the healthy group.

### *3.2. Correlation between fecal bacteria and blood lactate concentration*

Lactate-producing bacteria were investigated in all groups. *Bifidobacterium* spp. and *Enterococcus* spp. were more abundant in both DM groups than in the healthy group. Especially *Bifidobacterium* spp., which was higher in the L-DM group than in the N-DM group. *Lactobacillus* spp. was lower in the DM groups than in the healthy group (Figure 3).

### *3.3. Fecal bacteria associated with dysbiosis*

Three bacteria associated with dysbiosis were evaluated. *Blautia* mRNA expression was lower in the DM groups than in the healthy group, however, it was lower in the L-DM group than in the N-DM group. *Turicibacter* mRNA expression was significantly lower in the DM groups. When the lactate levels increased, the *Turicibacter* mRNA expression tended to decrease. The *Faecalibacterium* mRNA expression was lower in the DM groups than in the healthy group (Figure 4).

# 4. Discussion

In this study, we hypothesized that the blood concentration of lactic acid correlates with the changes in fecal microbiota of patients with IDDM. In order to investigate the changes in the microbiome according to blood lactate concentration, bacteria associated with lactate production, such as *Bifidobacterium* spp., *Enterococcus* spp., and *Lactobacillus* spp., were examined [9]. This study confirmed that *Bifidobacterium* spp. and *Enterococcus* spp. tend to increase and *Lactobacillus* spp. tend to decrease in diabetic patients compared to healthy counterparts. It is noteworthy that these findings are related to previously studied intestinal microbial flora [2, 10].

Brugman *et al*. investigated the fecal microbiome and found that *Lactobacillus* spp. and *Bifidobacterium* spp. populations exhibited lower microbial counts in rats with IDDM than in rats without diabetes [10]. Yang *et al*. found that the proportion of enterococci in the feces was higher in mice with IDDM [11]. Interestingly, Paterson *et al*. studied the changes in intestinal microflora with diabetes onset and progression and found that IDDM progression coincided with an increase in lactate-producing bacteria (i.e. *Lactobacillus* spp. and *Bifidobacterium* spp.) [12]. Barnett *et al*. proposed that diabetes-associated hyperlactatemia may be an early event in the time course of the disease [13]. In addition, Brouwers *et al*. reported increased lactate levels in patients with poorly controlled IDDM and glycogenic hepatopathy, implying that enhanced plasma lactate concentrations are part of the clinical spectrum of these diseases [6]. This study confirmed that even in

diabetic dogs, the intestinal fecal cells changed according to the blood lactate concentration. Although further research is needed on the factors that affect the gut microbiota in diabetic patients, this is the first study to report changes in lactate-producing bacteria in diabetics with elevated lactate levels.

In addition, lactate-producing microbiomes are known to play a major role in the formation of microflora in the intestinal tract, and by identifying *Blautia*, *Turicibacter*, and *Faecalibacterium* related to dysbiosis, it was confirmed how it affects other flora [9]. These findings indicate that the intestinal flora of diabetic patients changed according to their blood lactate levels, which means that the blood lactate concentration could be a major indicator of intestinal dysbiosis in diabetic patients.

It is well-known that the intestinal microbiota plays an important role in health by regulating digestion and supporting the immune system [14]. Therefore, various studies are being conducted to utilize intestinal bacteria in the treatment of chronic diseases such as metabolic diseases, cancer, inflammatory bowel disease, and autoimmune diseases [15]. In particular, an imbalance of the intestinal microflora is known to affect the immune system of patients with IDDM, which has been reported to exacerbate intestinal inflammation [16]. However, studies on the intestinal microflora in diabetic patients are still insufficient, and further research is necessary for the development of therapeutic agents that can target the intestinal microflora and their potential applications in diabetic patients.

While this study confirmed the presence of restrictive microbial bacteria, have not been able to determine whether dysbiosis is present in the feces. In addition, further studies are needed to determine whether the changes in microbial flora

observed in DM are a result of disease phenotype or have a causal relationship to pathophysiology. However, these findings are of great value as this was the first study to confirm changes in the fecal microbiota in terms of blood lactate concentration in diabetic dogs. These results will provide important basic data to help further the understanding of the microbiome in diabetic diseases in both veterinary and human medicine.

# 5. Conclusion

This study have confirmed that blood lactate levels are a major factor influencing changes in the fecal microflora of dogs with IDDM. These findings help further the understanding of the intestinal microflora of patients with IDDM.

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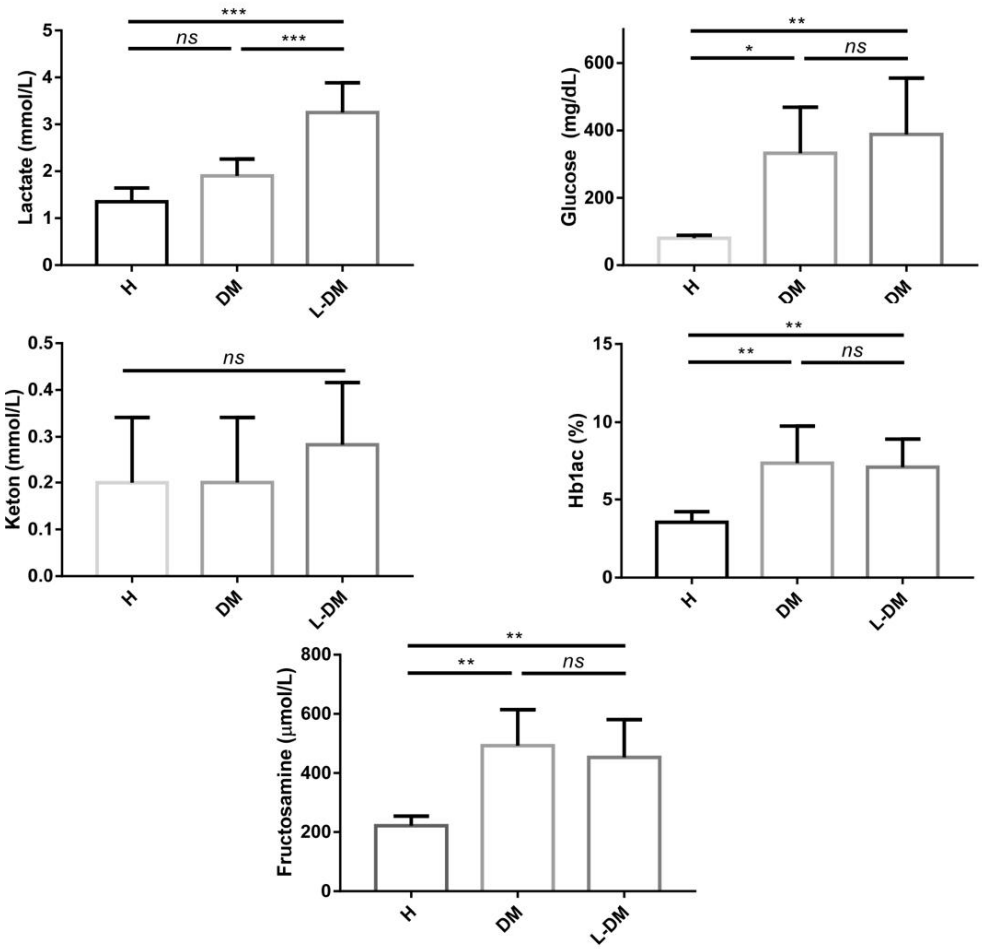


**Figure 1. Blood test of all groups.** Blood lactate concentrations (reference interval, 2.5 mmol/L or less). Blood glucose level (reference interval, 74.5 to 120 mg/dl). Blood ketone concentration (reference interval, 0.00 to 0.32 mmol/L). Blood fructosamine concentration (reference interval, 200 to 375 μmol/L). Blood Hb1ac concentration (reference interval, 5.1% or less). H = healthy (non-diabetes mellitus) dog group, N-DM = normal blood lactate concentration diabetes mellitus dog group. L-DM = Blood lactate concentration above the normal range diabetes mellitus dog group (*$P$<0.05, **$P$<0.01, ***$P$<0.001, ns (not significant) as determined by one-way ANOVA).

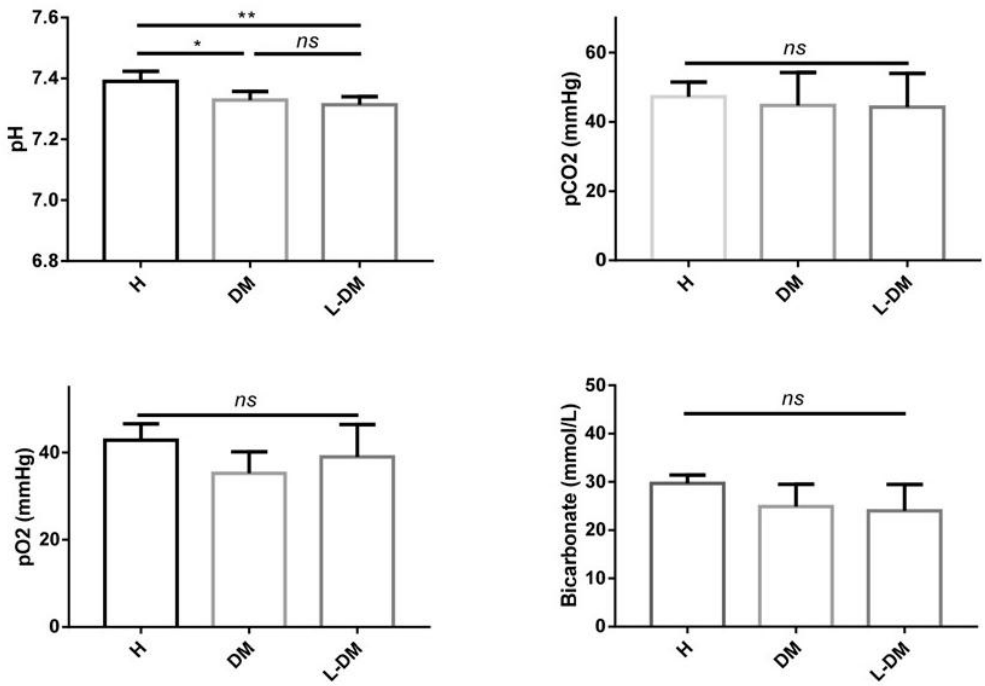


**Figure 2. Venous blood gas analysis in diabetes dogs.** Blood pH (reference interval range, 7.35 to 7.46). Blood pCO2 (reference interval, 32 to 43 mm Hg). Blood pO2 (reference interval, 80 to 105 mm Hg). Blood bicarbonate (reference interval, 18 to 26 mmol/L). All values calculated quantity with each dog temperature. H = healthy (non-diabetes mellitus) dog group, N-DM = normal blood lactate concentration diabetes mellitus dog group. L-DM = Blood lactate concentration above the normal range diabetes mellitus dog group (*$P$<0.05, **$P$<0.01, ns (not significant) as determined by one-way ANOVA).

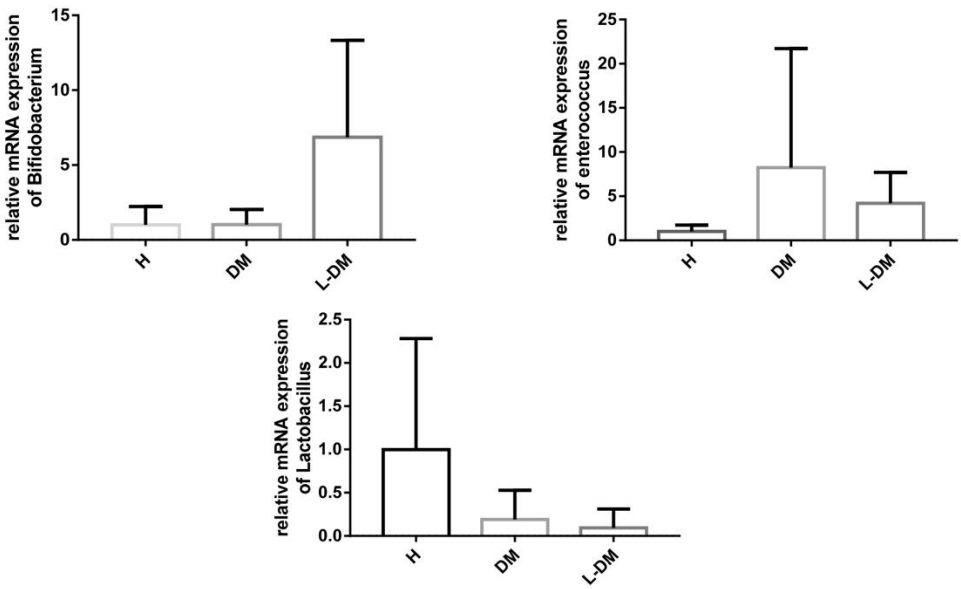


**Figure 3. Relative mRNA expression of lactate producing bacteria in feces of IDDM dogs.** Relative mRNA expression of *Bifidobacterium* spp.*, Enterococcus* spp.*, Lactobacillus* spp.. Relative mRNA expression is a proportion of universal mRNA expression for each Bacteria mRNA expression. H = healthy (non-diabetes mellitus) dog group, N-DM = normal blood lactate concentration diabetes mellitus dog group. L-DM = Blood lactate concentration above the normal range diabetes mellitus dog group.

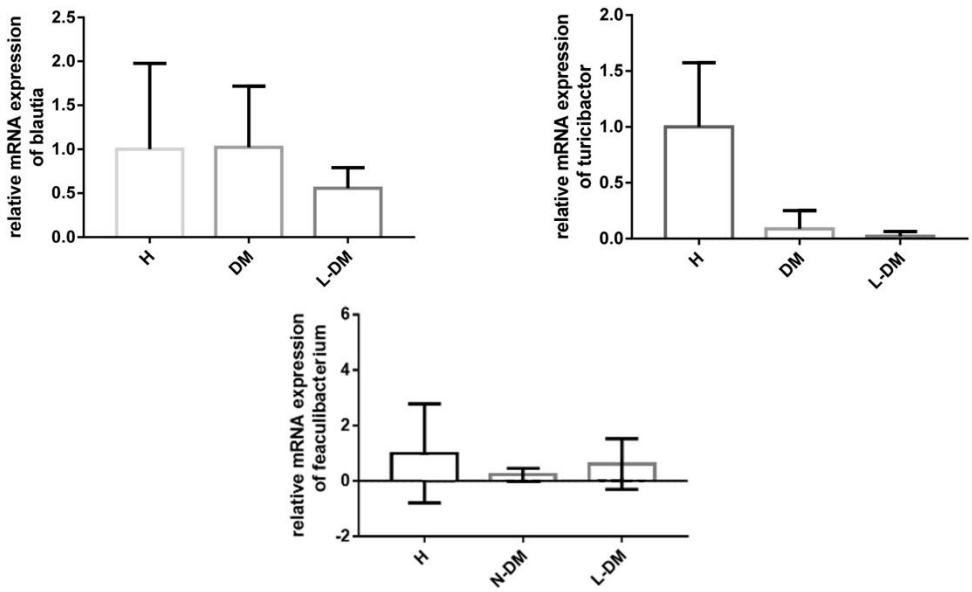


**Figure 4. Dysbiosis Index related bacteria relative mRNA expression in all groups.** Relative mRNA expression of *Blautia, Turicibacter. Faecalibacterium*. Relative mRNA expression is a proportion of universal mRNA expression for each Bacteria mRNA expression. H = healthy (non-diabetes mellitus) dog group, N-DM = normal blood lactate concentration diabetes mellitus dog group. L-DM = Blood lactate concentration above the normal range diabetes mellitus dog group.

**Table 1. Primer sequences used to detect gene expression of microbiota.**

| Genes | | 5'-3' primer sequences | References |
|---|---|---|---|
| Universal | Forward | CCT ACG GGA GGC AGC AGT | [9] |
| | Reverse | ATT ACC GCG GCT GCT GG | |
| *Faecalibacter* | Forward | GAA GGC GGC CTA CTG GGC AC | [9] |
| | Reverse | GTG CAG GCG AGT TGC AGC CT | |
| *Turicibacter* | Forward | CAG ACG GGG ACA ACG ATT GGA | [9] |
| | Reverse | TAC GCA TCG TCG CCT TGG TA | |
| *Blautia* | Forward | TCT GAT GTG AAA GGC TGG GGC TTA | [9] |
| | Reverse | GGC TTA GCC ACC CGA CAC CTA | |
| *Enterococcus* spp. | Forward | CCC TTA TTG TTA GTT GCC ATC ATT | [9] |
| | Reverse | ACT CGT TGT ACT TCC CAT TGT | |
| *Bifidobacterium* spp. | Forward | GGG TGG TAA TGC ATG | [17] |
| | Reverse | CAC CGT TAC ACC GGG AA | |
| *Lactobacillus* spp. | Forward | AGC AGT GAA TCT TCC A | [17] |
| | Reverse | ATT YCA CCG CTA CAC ATG | |

**Table 2. characteristics: including breed, age and sex of the 17 dogs.**

| Group | Breed | Years | Sex | Lactate | Concurrent Disease |
|---|---|---|---|---|---|
| Healthy | Beagle | 4 | IM | 1.1 | None |
| | Beagle | 3 | IM | 1.4 | None |
| | Beagle | 3 | IM | 0.9 | None |
| | Beagle | 4 | IM | 1.7 | None |
| | Beagle | 3 | IM | 1.5 | None |
| | Beagle | 2 | IM | 1.5 | None |
| N-DM | Maltese | 12 | MC | 1.6 | Urolithiasis |
| | Poodle | 10 | MC | 1.5 | History of Mast cell tumor (grade 2, low grade) resection |
| | Poodle | 9 | FS | 2.4 | None |
| | Spitz | 10 | MC | 2 | Tracheal collapse, Urolithiasis |
| | Maltese | 7 | MC | 2 | Chronic valvular heart disease ACVIM Stage B1 |
| L-DM | Maltese | 8 | MC | 3.1 | None |
| | Maltese | 11 | MC | 2.8 | Hyperadrenocorticism |
| | Pomeranian | 7 | IF | 3.7 | None |
| | Poodle | 13 | MC | 3 | None |
| | Yorkshire terrier | 13 | FS | 4.3 | None |
| | Chihuahua | 9 | MC | 2.6 | Hyperadrenocorticism |

IM (intact male), MC (male castrated), IF (intact femal), FS (female spayed)

# 7. 국문초록

## 인슐린 의존성 당뇨 환견의
## 마이크로바이옴과 혈중 젖산 농도의 상관관계

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수의학과 임상수의학 (수의내과학) 전공

장내 마이크로바이옴과 내분비 질환의 상관관계가 최근 관련 질환의 병리적, 임상적 접근의 중요한 열쇠로 주목받고 있다. 관련하여 인슐린 의존성 당뇨와 장내 세균 불균형 사이의 상관관계 또한 많은 연구결과에서 시사하고 있다. 그러나 이러한 연구는 insulin dependent diabetes mellitus(IDDM)과 장내 미생물 불균형의 인과관계와 원인 요인의 식별이 되지 않는다는 점에서 추가연구가 필요하다. 관리되지

않은 당뇨 질환은 사람뿐만 아니라 개에서 또한 혈중 젖산농도를 증가시킬 수 있다. 증가된 혈중 젖산농도와 장내 세균 불균형에 대한 보고는 연구된 바 있다. 따라서 본 연구에서는 혈중 젖산 농도와 관련하여 인슐린 의존성 당뇨병을 가진 개의 마이크로바이옴을 평가했다. 모든 실험 대상은 당뇨병이 있는 개와 건강한 개로 분류하였다. 자연적 발생 인슐린 의존성 당뇨를 가진 개(n = 11)와 건강한 개(n = 6)이 실험에 참여하였으며, 또한 인슐린 의존성 당뇨를 가진 개는 정상 혈중 젖산 수치를 가진 개(N-DM, n = 5)와 이상 젖산 수치를 가진 개(L-DM, n = 6)가 분류되었다. 모든 실험 대상 개들의 당뇨병 관리 혈액검사 지표를 측정하여 비교하였다. RT-qPCR을 이용해 젖산 생성균과 장내 세균 불균형 지표 균을 세균 범용 유전자와 비교하여 상대적 발현 경향을 확인하였다. 당뇨 환견과 건강한 환견의 *Enterococcus* spp.와 *Bifidobacterium* spp.의 상대적 mRNA 표현을 비교하였을 시 각각 6.23, 3.90 배 증가하는 경향을 확인하였다. 또한 혈중 젖산 농도가 증가한 당뇨 환견은 정상 젖산 농도를 가진 당뇨 환견에 비해 *Bifidobacterium* spp.의 상대적 mRNA 표현형이 3.90배 증가하는 경향을 확인하였다. 결론적으로, 혈중 젖산 농도는 IDDM을 앓는 개의 장 내 마이크로바이옴에 영향을 미치는 것으로 확인되었다. 이는 이전 진행되었던 장내 세균총 연구와 관련하여 한걸음 진보하였다는 의미가 있다. 또한 당뇨병 환자의 장내 세균총이 혈중 젖산 농도에 따라 달라짐으로 혈중 젖산농도가 당뇨 환자의 장내 세균

불균형 유발 요인에 주요하게 작용함을 확인하였다. 본 연구는 임상수의학에서 뿐만 아니라 인의에서도 당뇨병과 관련한 장 내 마이크로바이옴의 이해에 교량 역할을 할 것으로 사료된다.

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주요어 : 개, 인슐린 의존성 당뇨병, 마이크로바이옴, 젖산, 장 내 세균 불균형

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