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Determination Result of Colonic Lactobacillus in Healthy Adults

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Objectives: The aim of our study is to determine intestinal Lactobacillus of relativity healthy adults by colony forming units (CFU) and correlate the link between dairy consumption. **Methods:** This cross-sectional study was conducted in the city of Ulaanbaatar, Mongolia. A total of 80 healthy Mongolian adults, age 18-70 were recruited between April 2014 and June 2016 in the urban area. Participants self-collected freshly voided feces at home in a sterile container. **Results:** Of the total of 80 relatively healthy people from 18 to 70 years of age, there were 24 (30%) male and 56 (70%) female participants. By age group; 4 (5%) were age 15-24; 31 (38.7%) were age 25-34; 11 (13.7%) were age 35-44; 14 (17.5%) were age 45-54; 16 (20%) were age 55-64; 4 (5%) were over 65 years of age. The mean value of colonic Lactobacillus for relatively healthy adults was 1443×10^4 CFU/ml. Colony forming units of the gut lactobacillus were positively significantly associated with the intake of dairy products per week (r=.400, p<.001). **Conclusion:** Colonic Lactobacillus of relativity healthy adults was 1443×10^4 CFU/ml and the colony forming units of Lactobacillus was positively associated with the amount of dairy consumption and types of dairy products in the healthy people.

Keywords: Microbiota, Lactobacillus, Culture, Colony Forming Unit

Introduction

Lactobacillus, the major part of the lactic acid bacteria group, convert sugars to lactic acid, and constitute a significant component of the microbiota at a number of body sites, such as the digestive system, urinary system, and genital system[1]. Lactobacillus are a genus of Gram-positive, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria[1]. Lactobacillus accounts for 6% of the total bacterial intestine and 0.3% of the total body bacteria with 10^{4-7} CFU/ml in the intestine and 10^{10-11} CFU/ml in the colon[2]. Lactobacillus species produce lactic acids, hydrogen peroxides, bacteriocins, coagregation molecules and bio surfactants, acidolyns, acidophyllins and lactocidins etc[3]. Biosurfactants, lactic acids and bacteriocins pathogens interfere with cells, while hydrogen peroxide suppresses growth and coagulase molecules interfere with the distribution of the organism[4]. Lactobacillus spp. convert tryptophan to indole-3-aldehyde (I3A). Indole and I3A are agonists for the aryl hydrocarbon receptor (AhR), a transcription factor that regulates interleukin IL-22 expression, increases TH17-cell activity, and helps maintain intraepithelial lymphocytes. Indole upregulates the expression of tight junction proteins and modulates the expressions of pro- and anti-inflammatory genes in intestinal epithelial cells[5].

Many fermented foods, particularly yoghurt, contain high quantities of live bacteria, typically up to 10^9 CFU/g. These foods have been major contributors to the human diet since the Neolithic Era, yet our modern understanding of the impact of food-ingested bacteria on our resident gut microbiome remains limited[6]. To date, a majority of studies fail to identify significant modulations of the resident human gut microbiota upon consumption of fermented food[7-11].

Recently, host parameters such as age, gender and body mass index (BMI) are reported to be linked to the composition of the gut microbiota, and differences in dietary habits have been shown to affect the bacterial diversity of the human gut microbiota[12-15].

In Mongolia, a variety of traditional dietary habits and daily routines have developed. The typical Mongolian diet is characterized by high and frequent consumption of fermented dairy products. Dietary habits and gut microbiota studies of Mongolians have been limited to small population samples. Previous studies have focused on the profile of gut microbiota in patients with gastrointestinal disease[16,17].

This study features an investigation of the correlation between colonic lactobacilli and food consumption, especially dairy products consumed by the typical healthy Mongolian population in Ulaanbaatar. Therefore, the aim of our study is to determine the count of intestinal Lactobacillus in relativity healthy Mongolian adults and correlate these counts with dairy consumption.

Materials and Methods

Study design

This cross-sectional study was conducted using residents of the city of Ulaanbaatar, Mongolia. We studied healthy participants recruited at the University Hospital of the Mongolian National University of Medical sciences (MNUMS), National Center of Blood Transfusion in Ulaanbaatar, Mongolia.

Data collection and diet questionnaire

In this study, a total of 80 healthy Mongolian adults, age 18 to 72, were recruited between April 2014 and June 2016 in the urban area. A study error of 15% was assumed with a confidence interval of 1.96%. A sample size of 77 was calculated as adequate for this study. Each subject was asked to complete a questionnaire containing 67 questions about dietary habits of milk and dairy product consumption. This was a "Food pyramid" questionnaire based on the "Physiological norm of nutrition for population" as confirmed by minister's order №257 of the Mongolian Ministry of Health on 04, May 2017. The inclusion criteria were as follows: (1) participants ≥18 years old; (2) should be healthy by meeting the criteria based on decision of Mongolian Medical Academy conference on 22 May 2011; (3) Have a negative stool test for H.pylori antigen; (4) In the last month, no antibiotic or probiotic drug treatment. The following participants were excluded: (1) participants <18 years old; (2) In last month, subject received antibiotic or probiotic treatment; (3) Pregnancy or breastfeeding mother.

Evaluation of milk and dairy product dietary

Using daily serving size as defined by the food pyramid based on the "Physiological norm of nutrition for population" established by minister's order №257 of the Mongolian Ministry of Health on 04, May 2017, participants were classified into three groups of milk and dairy product consumption.

Level of milk and dairy products consumption was defined as: (a) If daily serving size is 0-50 ml, no consumption of milk and dairy products; (b) If daily serving size is 50-300 ml, a lower user group of milk and dairy products; (c) if daily serving size is above of 300 ml, a normal user group of milk and dairy products.

Based on type of daily milk and dairy products consumed, participants were classified as follows: milk user, protein-rich dairy product user, fermented dairy product user and butter rich dairy products user. Milk user group: cow's milk, horse's milk, sheep's milk, goat's milk. Protein-rich dairy products user group: curds, clabber, sour milk, dried curds, cream khuruud, milked dried curds, tsuraa, and khusam. Fermented dairy products user group: yogurt, fermented milk of horse and fermented milk of camel. Butter rich dairy products user group: clarified butter, white butter and butter of fermented milk of horse.

Fecal sampling

Participants self-collected freshly voided feces at home in a provided sterile container. The samples were kept refrigerated in the sterile container (Greetmed, Ningbo, China) until transported to the laboratory where they were processed within 2 hours and the fecal samples were stored for at -20^{0}C temperature until analyzed in the School of Biomedicine laboratory.

Culture for the isolation of lactobacilli

Fecal samples were then stored for 24 hours at 2 - 8^{0}C. Before analysis, the fecal samples were thawed at room temperature.

Fecal sample processing

The sample suspensions were prepared from the fecal samples under appropriate conditions in a biosafety cabinet. Suspensions were prepared by the addition of 1 ml of fecal sample to 9 ml of sterile selective broth. Suspensions were diluted ten times serially 10^1-10^6 and 100 ml was plated from each dilution on Rogosa agar and MRS (Man Rogosa Sharpe; Hardy Diagnostics, USA) with glacial acetic acid for selective growth of lactobacilli. The plates were incubated 48 hours at 37^0 Celsius under anaerobic conditions. Standard strains of L. acidophilus ATCC 314, L. casei ATCC 334, L. fermentum ATCC 9338 and L. gasseri ATCC 19992[4] used as growth control for lactobacillus species. After incubation, bacterial colonies cultured in the MRS selective media were counted by colony counter machine for each dilution (ProtoCOL3 Synbiosis, Division of Synoptics Ltd, USA). We tested catalase test activities of the colonies and they were all positive.

Statistical analysis

These data were processed with the Statistical Package for the Social sciences (IBM, SPSS Inc., version 22.0; Armonk, NY, USA). Statistical signification was defined as a p-value <.05. All data were tested using the Kolmogorov-Smirnov test and presented as the mean, standard deviation or median. We report the univariate and multiple regression models that include the relevant variables. After checking for normal distribution, an independent T test or an ANOVA was used for comparison among groups. For all comparisons, two-sided p-values <.05 were considered statistically significant with 95% confidence interval (CI). The Pearson correlation coefficient (for normal distributions) was used to assess the relationship of CFU/ml of lactobacillus with other values.

Ethical statements

The research methodology, recruitment procedure and consent forms were reviewed by the Medical Ethics Committee at MNUMS with approval issued on 2014.03.14 (№ 14-08/1A). Written Informed consent was obtained from all participants before inclusion in the study. Final ethical approval was issued by the Medical Ethics Committee (2018/D-10) at MNUMS on 2018.04.20.

Results

There were 80 study participants ranging from 18 to 70 years of age and relatively healthy. There were 24 (30%) male and 56 (70%) female participants (Table 1). 4 (5%) were age 15-24; 31 (38.7%) were age 25-34; 11 (13.7%) were age 35-44; 14 (17.5%) were age 45-54; 16 (20%) were age 55-64; 4 (5%) were over 65 years of age. Figure 2 shows the number of intestinal lactobacillus by age group. The mean count of intestinal Lactobacillus was statistically significant higher in participants 65 years old than found in participants 25-34 years old (p<.05).

Table 1. Participants characteristics (n=80)

Characteristics		N (%)
Sex		
	Male	24 (30.0)
	Female	56 (70.0)
Education		
	College	58 (72.5)
	High School	14 (17.5)
	Special Education	8 (10.0)
Disease Co-morbidity		
	Healthy	75 (93.7)
	Allergy	1 (1.3)
	Other diseases	4 (5.0)
Obesity		
	Yes	26 (32.5)
	No	54 (67.5)
Abdominal obesity		
	Yes	33 (41.3)
	No	47 (58.7)
Total		80 (100.0)

Data were presented as number (percentage); Obesity was determined by BMI classifications

The mean count of colonic Lactobacillus in relatively healthy adults was 1443×10^4 CFU/ml (Figure 1). As shown in Table 2, there was no statistically significant difference (p=.829) between males and females.

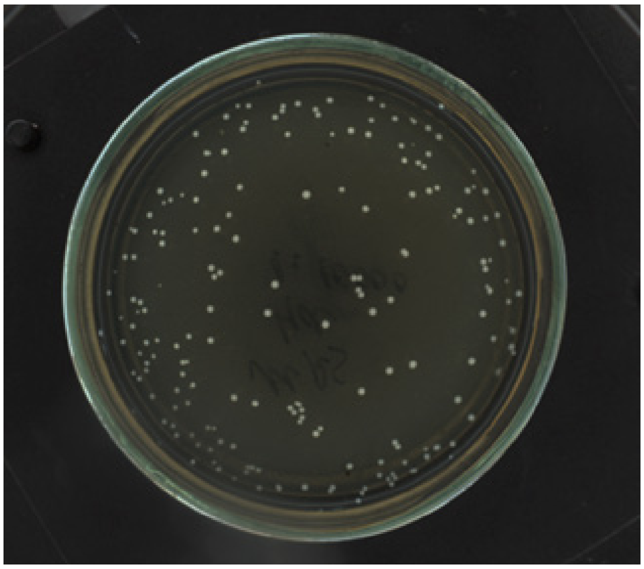

Figure 1. Lactobacilli colonies in selective outgrowth of lactobacilli. Lactobacillus is characterized by white/grey growths

In Table 3, 30.1% report an intake of dairy products 6-7 days per week and 69.9% report an intake of dairy products 1-5 days per week. According to the Health Ministry food pyramid that outlines healthy food choices for Mongolian individuals, 69.9% of the participants in our study did not consume recommended amounts of dairy products each day. In Table 3, there were significant differences in the total amount of gut lactobacillus species recorded between all groups based on dairy products intake per week (p=.02). Furthermore, the mean count of colonic lactobacilli of the intake of dairy products the 6-7 days per week consumption group is higher than the other two groups (all p<.05). In Table 4, CFU/ml of the gut lactobacillus were positively significantly associated with the dairy products intake per week (r=.400, p<.001).

Participants were divided into three groups; no consumption, inadequate consumption and adequate consumption of dairy products. In Table 3, their percentages were 37.5%, 58.8% and 3.8% respectively. The count of colonic lactobacillus was significantly different between no consumption and inadequate/adequate consumption of dairy products groups (p=.043).

In Table 3, 67.5% (n=54) of participants consumed unpasteurized milk. Among fermented dairy products users, their mean count of colonic lactobacillus was 2758.4×10^4 CFU/ml, which was significantly different than groups consuming other dairy products (p=.0001).

Table 2. Colonic Lactobacillus of healthy adults, 10^4 CFU/ml (by gender)

Gender	Lactobacillus m ± SD	95% CI	p-value
Male	1475 ± 791.6	251-3258	
Female	1429 ± 884.7	152-3161	.829[a]
Mean	1443 ± 853.1	152-3258	

[a]p value – Independent T test.

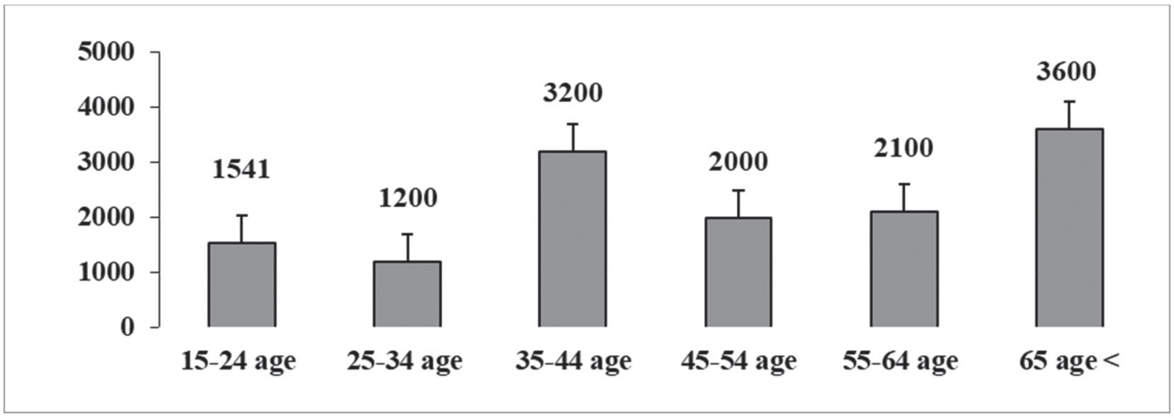

Figure 2. Colonic Lactobacillus 10^4CFU/ml (by age group)

Table 3. Results of multiple comparison between groups of dairy product consumption

	Participants (n=80)	Lactobacillus	p-value	Variable interval	p-value[b]
	n (%)	Mean ± SD			
Day of week				Dairy product frequency	
1 – 2 day	36 (45)	1263 ± 716.7		1-2 day x 3-5 day	.056
3 – 5 day	19 (23.75)	158 ± 843.0	.02[a]	1-2 day x ≥6 day	.010*
≥ 6 day	25 (31.25)	2088 ± 1024.5		3-5 day x ≥6 day	.040*
Size of dairy products on one time				Milk and dairy product type	
<50 ml (no consumption)	30 (37.5)	1330 ± 595.3	.043[b]	M x PDDP	.872
≥50 ml (inadequate and adequate)	50 (62.5)	1756 ± 1033.4		M x FaDP	.194
Milk and dairy product type				M x FeDP	.0001**
Milk	54 (67.5)	1378.9 ± 728.3		PDDF x FaDP	.042*
Protein dominated dairy product	10 (12.5)	1180.5 ± 307.6	.000[a]	PDDF x FeDP	.007*
Fatty dairy product	3 (3.8)	1878.0 ± 1195.4		FaDF x FeDP	.015*
Fermented dairy product	13 (16.2)	2758.4 ± 1016.7			

*p<.05; **p<.01; [a]ANOVA test; [b]Independent T test; M – Milk, PDDP – protein dominated dairy product, FaDP – fatty dairy product, FeDP – fermented dairy product

Table 4. Results of correlation analysis for the amount of some products to lactobacillus

	Age	Lactobacill, CFU/ml	Consumption of fiber, days	The intake of dairy product per week, days	Consumption of meat, days	Consumption of 500 grams salt, days
Age	1.00					
Lactobacilli, CFU/ml	-.040	1.00				
Consumption of fiber, days	.101	.150	1.00			
The intake of dairy product per week, days	.289**	.400**	.006	1.00		
Consumption of meat, days	-.165	-.020	-.270*	.127	1.00	
Consumption of 500 grams salt, days	.080	.036	.363**	.064	-.070	1.00

*p<.05; **p<.01

We investigated multiple comparisons between dairy product consumption groups, including frequency and type of dairy products. In multiple comparisons, the mean count of colonic lactobacilli was significantly different to group of ≥6 day consumption from other groups (all p<.05, Table 3). Also, the mean value of colonic lactobacilli was significantly different for groups using fermented dairy products and other groups (all p<.05, Table 3).

In Table 5, colonic lactobacilli counts were used as the dependent variable and the independent variables were age, consumption of 500 grams of salt, fiber, meat, dairy products intake per week, consumption amounts of dairy products and consumption amount of fermented dairy products. We showed that the dairy product intake per week (p<.0001), consumptions amount of dairy products and amount of fermented dairy products consumed (p<.0001, p=.002) were independent predictors of the colonic lactobacilli in univariate regression, respectively (Table 5). In the multivariate analysis model, the count of colonic lactobacilli was increased by 125.35 CFU/ml for each day increase of frequency the dairy products intake per week (β=125.35, 95% CI 24.97 – 168.36, p=.008). This increased to 351.03 CFU/ml in participants with high consumption amounts of dairy products (β=351.03, 95% CI 22.16 – 679.94, p=.037) and increased to 414.52 CFU/ml in participants with high consumption amounts of fermented dairy products (β=414.5, 95% CI 226.61 – 602.42, p=.000).

Discussion

Lactobacillus was detected at 10^8-10^{10} colonies of unit per gram in stool of a relatively healthy person, according to a study by Jens Wolter et al. in 2008[6]. The average number of lactobacillus from the relatively healthy people in our study was 1443×10^4 CFU/ml. That this count is lower than other studies may be caused by inappropriate antibiotic usage, insufficient dairy products usage, other dietary habits and dietary ingredients. However, this finding is similar to previous studies done by Elisabet Lonnermark and Agnes E Wold at Gothenburg University in Sweden in 2010[18]. In the Lonnermark and Gothenburg study, the mean count of lactobacilli in stool sample were 10^6 CFU/gm in control group. L.paracasei, L. gasseri, L.plantarum and L.rhamnosus were dominated in the control group[19].

In our study, the mean of intestinal Lactobacillus was statistically significant higher in participants above 65 years old than participants who were 25-34 years old. Similar results were confirmed in a cohort study thereafter by Le Roy that was done among relatively healthy Estonian people, in 2015[20]. The present study reported that L.paracasei, L.plantarum, L.salivarius, and L.helveticus were related to age. L.salivarius and L.helveticus were dominant among the participants under the age of 48. However, L.paracasei and L.plantarum were dominant among participants over the age of 65. Also, in culture studies of gut microbiota related to ageing, Mitsuoke found that bifidobacteria declined in elderly people compared with younger adults, whereas C.perfringes, Lactobacilli and Enterococci increased[21]. An increase in the number of intestinal lactobacilli is dependent on an increased amount of short-chain fatty acids, lactate, and essential amino acids contained in the stools. Amounts of intestinal lactobacilli were directly dependent on the activity of the host's metabolism[22]. Further research is needed to investigate the variation of normal intestinal microbial environments that depend on aging-related nutritional features, metabolic activity and associated disorders. There are different types of microorganisms per person, but adult's intestinal microbiotas are relatively stable in healthy people[3]. Metabolism, fermentation, methane processing, oxidizing phosphorus reaction and synthesis of lipopolysaccharide are reactions that combine many things[3]. Intestinal microflora fluctuates in the elderly which can cause decreased microflora coexistence and diversity, also the immune system of older adults may be suppressed. We have more to learn concerning the immune system, particularly the humoral immune system of the elderly which may be suppressed depending on the intestinal microbiome.

Among the questions examining dietary habits, our study showed that frequency of dairy products consumption was significantly correlated (p<.01) with the richness of Lactobacilli (Table 4). Researchers report that microbiological microorganisms affect eating habits and diet[23]. Feeding habits have important implications for intestinal microbiota. In previous studies, food type influenced gut microbiota, which consisted of more biliary metabolic dominant microflora in people with a meat rich diet[3,21]. In contrast, gut microbiota consisted more polysaccharides

Table 5. Regression of the colonic lactobacillus associated with consumption of dairy products

Independent	Colonic Lactobacillus			
	β	95% CI	p-value	VIF
Age	8.52	[-5.2 ; 22.2]	.220	1.15
Consumption of 500 grams salt	12.35	[-169.9 ; 194.5]	.893	1.10
Consumption of fiber	-64.64	[-153.9 ; 24.6]	.154	1.24
Consumption of meat	29.43	[-110.9 ; 169.8	.420	1.17
The intake of dairy product per week	125.35	[57.8 ; 193.0]	.000	1.24
Consumption size of dairy foods	340.04	[20.2 ; 641.4]	.000	1.36
Consumption size of fermented milk production	448.43	[242.4 ; 654.5]	.002	1.18
R square	0.201			
F value	2.45			

bacteria in people with a vegetable rich diet[23]. Mongolians used to have higher consumption of dairy products that related to a traditional nomadic lifestyle[24]. Nowadays consumption of dairy products is decreasing in Mongolia because of lifestyle changes resulting from urban living[24].

When compared to the food pyramid recommendations for Mongolian individuals, 69.9% of participants in our study did not consume an adequate amount of dairy products every day. There were huge differences in the total amount of gut lactobacillus species between participants who consume milk 6-7 days per week and those who consume milk less than 5 days per week. The food pyramid suggests that total food consumption per day must include 10% dairy products which equals 125-250 ml milk. Also, dietary need for dairy products must be adjusted according to the age group. According to the food pyramid, 250-375 ml milk (30-45 mg curds) of dairy products should be consumed by children 4-10 years of age. It is recommended that children 11-17 years of age, adults and older people consume 200-400 ml milk or 30-60 mg curds of dairy products. Additionally, it is recommended that pregnant women consume 500 ml milk or 240 mg curds of dairy products per day. In our study, 30.1% of total participant have an intake of dairy products 6-7 days per week and 69.9% of total participants consume dairy products 1-5 days per week.

In this study, 37.5% of participants do not consume milk, 58.8% do not consume adequate milk and only 3.8% consume adequate milk as recommended for Mongolian adults. Our findings show that 96.2% of study participants do not consume adequate amounts of milk as shown by the food pyramid. In the future, we see a need for study of how seasonal usage of dairy products correlates with colonic lactobacillus.

This study had important limitations. First, the sample size is small compared to other studies. Another limitation of our study is that we did not measure lactobacillus species by PCR technology as a result of limitations of our detection means and technology. Thirdly, even though we detected lactobacillus via culture, there are many additional lifestyle factors that might affect study outcomes were not available, such as dietary fiber, alcohol use, unreported antibiotic usage, seasonal changes and other lifestyle variations.

In conclusion, Colonic Lactobacillus of relativity healthy adults were 1443×10^4 CFU/ml and the colony forming units of Lactobacillus was positively associated with the amount of dairy consumption and types of dairy products consumed by healthy people.

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