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RESEARCH ARTICLE

The Effect of Total Mix Ration with Xylitol Supplementation on *In Vitro* Ruminal Total Gas and Methane Production, Digestion Values, Organic Acids, Ammonia-Nitrogen, and the Number of Total Protozoa in Dairy Cattle

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Abstract: The study aimed to determine the effect of xylitol, added to the dry period total mix ration (TMR) of dairy cattle, on *in vitro* rumen fermentation, total gas production, methane production, estimated digestion values, organic acids and ammonia-nitrogen contents and the number of total protozoa. Xylitol was added to dairy cattle TMR at different rates (0%, 2% 4%, and 8%; dry matter basis). The xylitol supplementations at 2 and 4% did not affect *in vitro* total gas production, *in vitro* methane production, metabolizable energy (ME) and organic matter digestibility (OMd) values (P>0.05). However, 8% xylitol supplementation decreased *in vitro* total gas production, *in vitro* methane production, ME and OMd (P<0.05). The molarities of total volatile fatty acid (TVFA) percentages of acetic acid (AA), propionic acid (PA) and butyric acid (BA) in TVFA, ammonia-nitrogen concentration, and the number of total ciliate protozoa of the *in vitro* rumen fluid of xylitol supplementations at 2%, 4%, and 8% were similar to those of control TMR (P>0.05). The iso-valeric acid (IVA), iso-butyric acid (IBA) and valeric acid (VA) percentages in TVFA of *in vitro* rumen fluid linearly decreased with xylitol supplementation, especially 8% xylitol supplementation (P<0.05). Besides, 2% and 4% xylitol supplementations to dairy cattle TMR numerically increased the concentration of ammonia-nitrogen and the number of total ciliate protozoa in *in vitro* fermentation fluid (P>0.05). Consequently, the supplementation of 2% and 4% xylitol to dairy cattle ration did not affect the *in vitro* rumen fermentation (total gas production, methane production, estimated digestion values, organic acids and ammonia-nitrogen parameters). However, 8% supplementation xylitol to dairy cattle ration had the potential to affect the before-mentioned *in vitro* ruminal fermentation parameters adversely.

Keywords: *Dairy cattle, In vitro gas production, Rumen fermentation, Xylitol*

Ksilitol İçeren Süt Sığırı Karma Rasyonunun *İn vitro* Ruminal Total Gaz ve Metan Üretimi, Sindirim Değerleri, Organik Asit ve Amonyak-Azotu Parametreleri İle Total Protozoa Sayısına Etkisi

Öz: Çalışmanın amacı, süt sığırlarının kuru dönem toplam karma rasyonuna (TKR) ilave edilen ksilitolün *in vitro* rumen fermantasyonu, toplam gaz üretimi, metan üretimi, tahmini sindirim değerleri, organik asitler ve amonyak-azotu içerikleri ve toplam protozoa sayısı üzerindeki etkisini belirlemektir. Ksilitol, süt sığırı TKR'sinin kuru maddesine (KM) farklı oranlarda (%0, %2, %4 ve %8) ilave edilmiştir. Süt sığırlarının kuru dönem TKR'sine %2 ve %4 ksilitol ilavesi, kontrol TKR'sine göre *in vitro* toplam gaz üretimi, *in vitro* metan üretimi, metabolik enerji (ME) ve organik madde sindirimi (OMd) değerlerini değiştirmemiştir (P>0.05). Bununla birlikte, %8'lik ksilitol ilavesi *in vitro* toplam gaz üretimi, *in vitro* metan üretimini, ME ve OMd değerlerini azaltmıştır (P<0.05). Süt sığırlarının kuru dönem TKR'sine (KM'de) %2, %4 ve %8 ksilitol ilavesi *in vitro* rumen sıvısının toplam uçucu yağ asiti (TUYA) molaritesi, TUYA içindeki asetik asit (AA), propiyonik asit (PA) ve bütirik asit (BA) oranı, amonyak-azotu konsantrasyonu ve toplam siliatalı protozoa sayısı, kontrol TKR'sinin değerleriyle benzer olduğu belirlenmiştir (P>0.05). *In vitro* rumen sıvısının TUYA'deki iso-valerik asit (IVA), iso-bütirik asit (IBA) ve valerik asit (VA) oranları ksilitol ilavesiyle (özelllikle %8 ksilitolde) linear olarak azalmıştır (P<0.05). Ayrıca, süt sığırı TKR'sine %2 ve %4 ksilitol ilavesi, *in vitro* fermantasyon sıvısının amonyak-azot konsantrasyonunu ve toplam siliat protozoa sayısını rakamsal olarak artırdığı gözlenmiştir (P>0.05). Sonuç olarak, süt sığırı rasyonlarına %2 ve %4 ksilitol ilavesinin *in vitro* rumen fermantasyonunu (toplam gaz üretimi, metan üretimi, tahmini sindirim değerleri, organik asitler ve amonyak-azot parametreleri) etkilememiştir. Bununla birlikte, süt sığırı rasyonlarına %8 ksilitol ilavesinin, daha önce bahsedilen *in vitro* ruminal fermentasyon parametrelerini olumsuz etkileme potansiyeline sahip olduğu belirlenmiştir.

Anahtar sözcükler: *Süt sığırı, In vitro gaz üretimi, Rumen fermentasyonu, Ksilitol*

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Introduction

The transition period is the most critical physiological stage in dairy cattle health, production, reproduction, and profitability three weeks before and three weeks after parturition. During this period, decreased feed intake due to decreased rumen capacity, increased energy and nutrients needs for colostrum and milk synthesis in late pregnancy cause negative energy balance (NEB) and micronutritient deficiencies in dairy cattle [1-3]. Several metabolic (hypocalcaemia, displacement of the abomasum, fatty liver syndrome, and ketosis etc.), infectious and reproductive disorders such as mastitis, dystocia, retained placenta and metritis reported during the early lactation period in high producing herds causes significant economic losses [1,4].

In recent years, the use of glucogenic substances has been increasing in high-producing dairy cows to prevent hepatic lipidosis and ketosis and reduce energy and glucose deficit in the transition period [5]. It has been reported that the use of additives such as glycogen, oils, glycerol, propylene glycol (PG), propionates, monensin, methionine, lysine, choline, niacin, biotin, sodium borate and conjugated linoleic acid (CLA) in the peripartum period may be beneficial in overcoming the periparturient period without any problems [6-8].

Xylitol is an antiketotic, 5-carbon sugar alcohol that stimulates insulin secretion and is used to treat diabetes in humans and cattle with ketosis in Japan. Xylitol can be metabolized as it enters the pentose phosphate cycle even without insulin [9-11]. Sakai et al.[11] determined that blood glucose and insulin response were more effective than glucose, when xylitol treated cattle with ketosis, intravenously. They also reported that xylitol application is beneficial in the treatment of ketosis due to the disappearance of urinary ketone bodies, increased feed consumption and clinical improvement. Toyoda et al.[4] administered xylitol intravenously to healthy and ketotic cattle and determined that it caused a gradual increase in insulin level in ketotic cattle, while a temporary increase in insulin secretion occurred in healthy cattle. Hamada et al.[12] reported that blood glucose levels increased, and ketone bodies decreased when they administered 100 g of xylitol intravenously in cattle with ketosis. One study examined the fattening performance in calves with oral use of xylitol, although there are reports of intravenous use of xylitol in dairy cattle. Although there is no difference in feed consumption in calves given xylitol, glucose and polylol, it has been reported that xylitol is very effective in weight gain [13].

The hypothesis of this study is not the adverse effects on the *in vitro* rumen fermentation of different doses of xylitol, which is an alternative to energy sources in the diet, such as propylene glycol, in the dry period and fresh period (transition period) of dairy cattle. The aim of the present study was determined to be the effect of different doses of xylitol added to the diet on the *in vitro* rumen fermentation, total gas production, methane production, estimated digestion values, organic acids and ammonia-nitrogen parameters and the total number of protozoa.

Material and Methods

Sample Features

Xylitol (Ksilitol, Nustil®, İstanbul), which is a natural source of birch and beech bark, was supplemented at different rates (0, 2, 4, and 8% dry matter basis) to dairy cattle TMR. Xylitol ratios were determined by modifying the method of Lister and Smithard [14].

Composition of the Dairy Cattle Total Mix Ration

The dairy cattle total mix ration (TMR), which was used in the *in vitro* gas production technique, was composed of 25% corn silage, 13% wheat straw, 20% dried alfalfa herbage [with 17-19% crude protein (CP), 40-44% neutral detergent fiber (NDF)], 16% barley, 8% sunflower meal (28% CP), 9% cottonseed meal (28% CP), 8% wheat bran and 0.1% magnesium sulfate (for anionic diet). The crude protein (CP), net energy lactation (NEL), neutral detergent fiber (NDF) and non-fiber carbohydrate (NFC) content of the dairy cattle ration were 14.2% DM,1.34 Mcal/kg DM, 45.6% and 32.4%, respectively. These rates indicate the dry matter percentages of the feedstuffs.

The Determination of In Vitro Ruminal Digestion Potential and Rumen Fluid Collection

The dairy cattle TMR, which included corn silage, wheat straw, alfalfa herbage, crushed barley grain, sunflower meal, cotton seed meal and wheat bran were used as control. The dairy cattle TMR (control) was prepared in a composition to meet the energy and nutrient requirements of the *Holstein* cattle in the last two months of pregnancy (dry period) [15].

The Determination of In Vitro Total Gas Production, Methane Production, and Estimated Digestion Values

The *in vitro* cumulative total gas production was recorded at 24 h. After 24 h of incubation, the total gas volume was recorded from the calibrated scale in the *in vitro* glass fermenter [16]. After reading the total gas volume, the methane volume in total gas was determined with the infrared methane analyzer (Sensor, Europe GmbH, Erkrath, Germany). The metabolizable energy (ME) and organic matter digestibility (OMd) contents of the dairy cattle TMRs with 0%, 2%, 4%, and 8% Xylitol supplementation as dry matter (DM) were calculated using the equations by Menke and Steingass [17] and Blümmel et al.[18] as follows:

(ME (MJ /kg DM) = 2.20 + 0.136 × Gas24h + 0.057 × CP), (OMd (g/kg DM) = 14.88 + 0.889 × Gas24h + 0.45 × CP + 0.0651 × A). Gas24h = 24 h net gas production (mL/200 mg), CP = Crude protein (g/kg DM), CA = Crude ash content (g/kg DM), EE = Ether extract (g/kg DM).

Determination of Total Ciliate Protozoa Number

At the end of the incubation period, the content of *in vitro* fermentation fluid in glass syringes was used for counting total protozoa [19].

Determination of pH and Ammonia Concentration

The pH value of the filtered *in vitro* fermentation fluid was determined using a digital pH meter (Mettler Toledo S220, Switzerland). The ammonia concentration (mg/L) in the *in vitro* fermentation fluid was determined using a commercial ammonia assay procedure (Megazyme, K-AMIAR 02/20, Wicklow, Ireland) [20].

Determination of Volatile Fatty Acids in the In Vitro Fermentation Fluid

The total gas volume at 24 h of *in vitro* incubation was recorded, and 10 mL of the ruminal fermentation fluid in the glass fermenter was collected into Falcon tubes. The volatile fatty acid (/organic acid) (acetic-AA, propionic-PA, butyric-BA, iso-valeric-IVA, iso-butyric- IBA, valeric-VA and hexanoic - HA acids) molarities in the *in vitro* ruminal fermentation fluid were determined using the gas chromatography (GC) device (Thermo Trace 1300, Thermo Scientific, Waltham, MA, USA). The GC device was equipped with a flame ionisation detector (FID) and a polyethylene glycol column (length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm) (TG-WAXMS, Thermo Scientific, Waltham, MA, USA). The device was operated according to the procedure described by Ersahince and Kara [21]. The percentages of induvial volatile fatty acids in total volatile fatty acids (TVFA), whose molarity was determined by the Xcalibur programme (Thermo Scientific, Waltham, MA, USA), were calculated.

Statistical Analyses

The obtained data were statistically analysed using SPSS 17.0 software (IBM Corp., Armonk, USA). One-way analysis of variance was conducted for variables tested in different doses ofxylitol supplementation. Data were analyzed using the following statistical model:

$$Yij = \mu ij + Si + ei$$

where: Yij = the general mean for each parameter investigated; µ = the mean of xylitol supplementation for each tested parameter; Si = the i th effect of xylitol supplementation on the observed parameters; ei = the standard error The significance of differences in means was revealed using Tukey's multiple range test at $P<0.05$.

Results

The xylitol supplementations at 2% and 4% to dry matter (DM) of dry period TMR of dairy cattle did not change *in vitro* total gas production, *in vitro* methane production, ME and OMd values according to those of control TMR ($P>0.05$). However, 8% xylitol supplementation led to decrease *in vitro* total gas production, *in vitro* methane production, ME and OMd ($P<0.05$) *(Table 1)*.

The molarities of TVFA and percentages of AA, PA, and BA of the *in vitro* rumen fluid of xylitol supplementations at 2%, 4%, and 8% to dairy cattle dry period TMR were like those of control TMR ($P>0.05$). The percentages of IVA, IBA, and VA of *in vitro* rumen fluid decreased with xylitol supplementation, especially 8% xylitol supplementation ($P<0.05$) *(Table 2)*.

The ammonia-nitrogen concentration and number of total ciliate protozoa of *in vitro* fermentation fluid did not change with xylitol supplementation to dairy cattle TMR ($P>0.05$). Besides, 2% and 4% xylitol supplementation to dairy cattle TMR numerically increased the concentration of ammonia-nitrogen and the number of total ciliate protozoa of *in vitro* fermentation fluid ($P>0.05$) *(Fig. 1, Fig. 2)*.

Discussion

Xylitol is a sugar alcohol used as a sweetener for diabetics and also for other purposes (e.g., in chewing gum). Xylitol has attracted global demand mainly due to its insulin-independent metabolism, anti-carcinogenicity, sweetening

Table 1. *In vitro total gas production, methane production and estimated digestion values of dairy cattle TMR with Xylitol supplementation*

Parameters	Xylitol Supplementation to Dairy Cattle TMR*				SEM	P Value	
	0%	2%	4%	8%		L	Q
In vitro total gas production	41.77^{a}	41.63^{a}	36.33^{ab}	29.80^{b}	1.78	0.004	0.179
In vitro methane production	11.57^{a}	12.01^{a}	9.64^{ab}	8.50^{b}	0.52	0.007	0.266
In vitro ME	8.68^{a}	8.66^{a}	7.94^{ab}	7.05^{b}	0.24	0.004	0.181
In vitro OMd	58.83^{a}	58.70^{a}	53.99^{ab}	48.19^{b}	1.58	0.004	0.184

** Xylitol supplementation to DM of dairy cattle TMR, total gas production is as mL/0.2 g DM, Methane production is as mL/0.2 g DM;* ***TMR:*** *total mix ration;* ***OMd:*** *Organic matter digestibility;* ***ME:*** *Metabolizable energy;* ***SEM:*** *Standard error of means*

Table 2. *Ruminal volatile fatty acids of in vitro fermentation for dairy cattle TMR with xylitol supplementation*

Parameters	Xylitol Supplementation to Dairy Cattle TMR*				SEM	P Value	
	0%	2%	4%	8%		L	Q
TVFA, mmol/L	99.86	96.78	94.63	92.69	1.36	0.162	0.385
% values of volatile fatty acids in TVFA							
Acetic acid	70.29	70.22	70.82	70.66	0.11	0.065	0.586
Propionic acid	13.56	13.65	13.43	13.87	0.07	0.782	0.057
Butyric acid	12.21	12.57	12.32	12.27	0.06	0.933	0.181
Iso-valeric acid	1.60^{a}	1.37^{ab}	1.27^{b}	1.18^{b}	0.05	0.002	0.024
Iso-butyric acid	0.91^{a}	0.81^{b}	0.77^{b}	0.73^{b}	0.02	0.002	0.007
Valeric acid	1.12^{a}	1.03^{ab}	1.03^{ab}	0.95^{b}	0.02	0.025	0.018
Hexanoic acid	0.31	0.33	0.34	0.31	0.005	0.052	0.682

** Xylitol supplementation to DM of dairy cattle TMR,* ***TVFA:*** *total volatile fatty acids are sums of acetic acid + propionic acid + butyric acid + iso-valeric acid + iso-butyric acid + valeric acid + hexanoic acid;* ***TMR:*** *Total mix ration;* ***SEM:*** *Standard error of means*

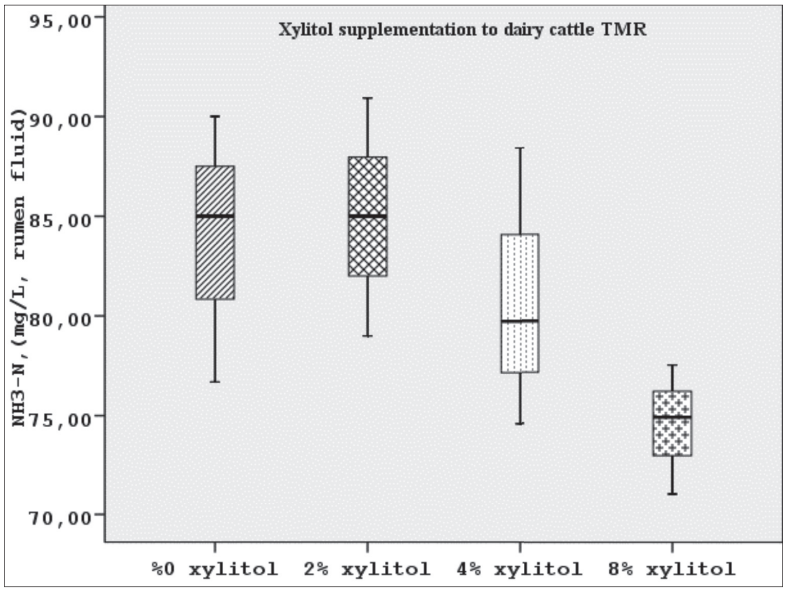
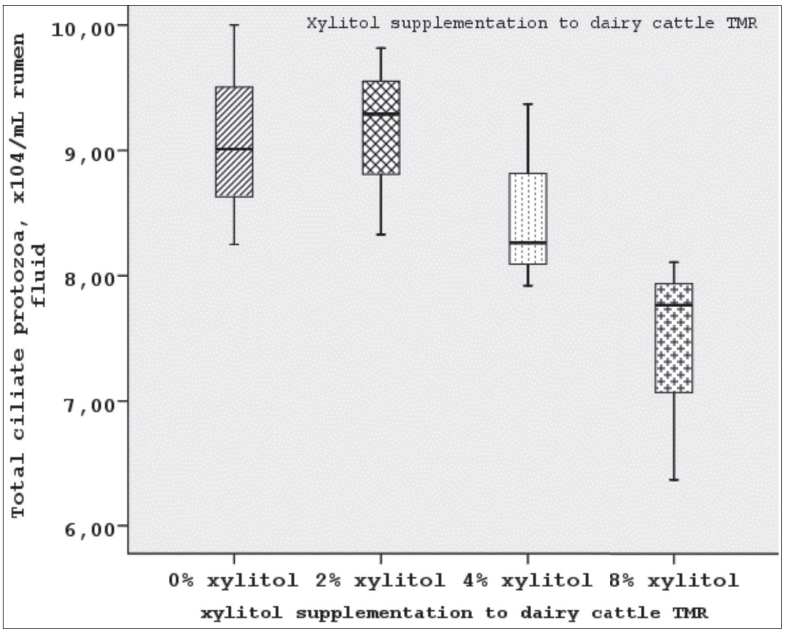


Fig 1. Ammonia-nitrogen concentration of ruminal fluid the *in vitro* fermentation for dairy cattle TMR with Xylitol supplementation (P value; Linear: 0.529 and Quadratic: 0.147)

Fig 2. Total ciliate protozoa number of ruminal fluid the *in vitro* fermentation for dairy cattle TMR with xylitol supplementation (P values; Linear: 0.815 and Quadratic: 0.188)



power similar to sucrose, and pharmacological properties [22]. However, there is not enough information about the efficacy of xylitol in rumen fermentation in the literature and the dose to be added to the feed. In the present study, the xylitol supplementations at 2% and 4% to DM of dry period TMR of dairy cattle did not change *in vitro* total gas production, *in vitro* methane production, ME and OMd values according to those of control TMR demonstrated that did not change normal ruminal fermentation of these doses of xylitol. However, in the presented study, it was understood that the addition of 8% xylitol to the TMR can reduce *in vitro* ruminal fermentation values. The results of the study show that the high use of this prebiotic sugar alcohol (xylitol) in the rumen may have negative effect. The fact that there is no study on the effect of the use of xylitol in the diet on the fermentation values in ruminants causes the study results not to be discussed with ruminants. Xu et al.[23] stated that xylitol significantly enhanced the level of butyrate synthesizing bacteria such as *Clostridium* and *Phascolarcto bacterium* in the *in vitro* colonic simulation of human. The same researchers showed that xylitol increased the production of propionic acid and butyrate in the *in vitro* colonic simulation of humans [23].

It is vital to reduce the methane emissions of ruminants (especially in dairy cattle enterprises) due to their undesirable contribution to global warming. In recent years, there have been studies on this subject with different additives or alternative feedstuffs materials. The aim here is to reduce methane production without adversely affecting rumen fermentation and digestion in the rumen (without adversely affecting the utilization level of energy and nutrients in the feed) [24-26]. In the presented study, despite reducing methane production with the addition of 8% xylitol, it is not desired that the *in vitro* total gas production and ME and OMd values be also reduced. In this respect, using of xylitol as an anti-methanogenic additive is not recommended at these doses.

In the study, TVFA, AA, PA, BA, IBA, VA and IVA molarities of the *in vitro* rumen fluid were compatible with reference values [21,27]. The molarities of TVFA, percentages of AA, PA and BA of the *in vitro* rumen fluid of xylitol supplementations at 2, 4 and 8% to DM of dry period TMR of dairy cattle were like those of control TMR. In another study, the molarity of BA increased with increasing xylitol supplementation doses of the *in vitro* ruminal batch culture (at 0.85, 2.13, and 4.25 g/L concentrations) for 12 h [28]. Researchers demonstrated that these two lower xylitol concentrations decreased the percentage of PA in TVFA [28]. Using a ration consisting of roughage, the researchers differed from our study results [28]. The total mix ration of dairy cattle was used in the current study, and it was rich in soluble and digestible carbohydrates. In rumen fermentation, soluble and easy fermentable carbohydrates (pentoses; L-arabinose, D-ribose, and D-xylose, pentitols; L-arabinitol, ribitol, and xylitol) have fermented as firstly and, they have demonstrated high gas production and fermentation kinetics. Then the digested carbohydrates (such as starch) have fermented, followed by structural carbohydrates such as cellulose and hemicellulose [15]. The linearly decreasing of IVA, IBA and VA percentages in the TVFA of *in vitro* rumen fermentation fluid in the present study can show the potential of this polyol compound to alter rumen fermentation in a dose-dependent manner.

Some of the nitrogenous compounds (true proteins and other nitrogen-containing compounds) in the rumen are decomposed into ammonia in the rumen. Some of this ammonia (in the presence of sufficient alpha keto-acids) is absorbed into the microbial protein. A part of it is absorbed from the rumen wall and comes back to the rumen with saliva [15,29]. However, proteinaceous compounds passing through the rumen and microbial proteins produced in the rumen can be taken from the intestines as amino acids. In the presented study, the fact that both ruminal ammonia nitrogen and ruminal protozoa levels did not change shows that these levels of xylitol used in TMR do not have a negative effect on protein metabolism and milk yield.

In conclusion, it was thought that the use of xylitol compound as an energy source in dairy cattle rations did not have a negative effect on ruminal fermentation at 2% and 4% doses and these doses should be applied in the *in vivo* feeding trials.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author.

Funding Support

There is no funding source.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

The rumen contents used in this study were obtained from the slaughterhouse. Ethics committee approval is not required for this study because of performing *in vitro* in the laboratory.

Author Contributions

YS and ÖA: the hypothesis of this study; ÖA and KK: work management, article writing; KK, SEÖ, SY and MAÖ: experimental procedure follow-up; YS, ÖA, KK, SEÖ, SY and MAÖ: literature review, review of results, final decision.

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