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Effects of various inulin levels on *in vitro* digestibility of corn silage, perennial ryegrass (*Lolium perenne* L.) and common vetch (*Vicia sativa* L.)/oat (*Avena sativa* L.) hay

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Abstract

The aim of this study was to investigate the effects of various inulin levels on *in vitro* true dry matter digestibility ($IVTD^{DM}$) and *in vitro* neutral detergent fibre digestibility ($IVTD^{NDF}$) of corn silage (CS), perennial ryegrass (PR), and common vetch/oat hay (VO). Inulin was added to the fermenter at concentrations of 0 (CSC, PRC, VOC), 100 (CS100, PR100, VO100), 200 (CS200, PR200, VO200), and 300 (CS300, PR300, VO300) mg/litre of total culture fluid using an *in vitro* Daisy^{II} incubator. Each fermenter contained 1600 ml buffer solution and 400 ml rumen fluid. The $IVTD^{DM}$ and $IVTD^{NDF}$ were determined with a Daisy^{II} incubator and rumen fluid obtained from three cannulated Karayaka rams. The $IVTD^{DM}$ values (%) for PRC, PR100, PR200, and PR300 were 70.06 ± 1.133, 73.21 ± 4.153, 70.36 ± 0.506, and 66.69 ± 1.317, respectively. The effects of various inulin levels on $IVTD^{DM}$ and $IVTD^{NDF}$ values of PR were significant (P <0.05). The $IVTD^{DM}$ and $IVTD^{NDF}$ values for CS and VO were not significantly (P >0.05) different. Among the treatments, supplementation of inulin to CS and VO did not have a significant (P >0.05) effect on $IVTD^{DM}$ and $IVTD^{NDF}$ values. The high dose of inulin (PR300) reduced $IVTD^{DM}$ and $IVTD^{NDF}$, whereas PR100 showed a statistically significant (P <0.05) increase on $IVTD^{DM}$ and $IVTD^{NDF}$. However, *in vivo* studies with PR may be required to show the effects of various levels of inulin supplementation to support the IVTD findings of the current study.

Keywords: Inulin, *in vitro* true digestibility, hay, silage
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Introduction

Inulin is a stored complex carbohydrate of the fructans group in plants. It is estimated that inulin is produced by more than 36000 species of plants, with a large proportion of commercial inulin being obtained from the roots of chicory plants (Flickinger *et al*., 2003). Inulin is a linear multi-dispersible carbohydrate containing fructosyl-fructose residues with β-2,1-linkages (Waterhouse & Chatterton, 1993). Inulin is used as a feed additive for many animals, including rabbits, pigs, poultry, dogs, and cats (Samanta *et al*., 2013; Kozłowska *et al*., 2016). Kozłowska *et al.* (2016) reported that inulin increases performance and carcass weight in poultry. Alzueta *et al*. (2010) reported a significant effect of inulin on the digestibility of most amino acids and major fatty acids.

Traiyakun & Paengkoum (2013) indicated that inulin is resistant to enzymatic hydrolysis, especially cellulose and hemicellulase enzymes. Furthermore, inulin from chicory and Jerusalem artichoke has potential to improve nitrogen utilization.

The rates of organic acid formation of inulin are slower than those of glucose and phlein (product of orchard grass fructan). The rates of substrate conversion for fructans tend to be faster than that of inulin (Hall & Weimer, 2016). Kasperowicz & Michalowski (2002) report that sucrose was hydrolysed at a significantly lower rate than Timothy grass fructan, whereas inulin was degraded at the lowest rate of the three carbohydrates tested. The most important determinants of the nutritive value of forage for ruminants are its chemical composition, digestibility, and feed consumption (voluntary intake) (Khazaalet *al*., 1993). Corn silage, VO and PR are commonly used roughages in ruminant nutrition. *In vivo* digestibility studies are

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costly and time consuming, whereas *in vitro* digestibility studies are cheaper and results are produced more rapidly. Creating the conditions of rumen under laboratory conditions allows the in vitro determination of the digestibility of feed. This technique is simple and is commonly used in the analysis of conventional ruminant feeds. The nutritive values of CS, VO and PR are usually determined by the rate and extent of degradation of the organic matter by rumen fluid. In addition, in vivo and in vitro studies need to be performed with ruminants and with rumen fluid to further investigate the potential of inulin to improve feed conversion rates. The aim of study reveals the effect on digestibility because inulin is degraded more slowly than other carbohydrates in rumen. Few data are available on IVTD and neutral detergent fibre digestibility of inulin. Therefore, the objective of the present study was to investigate the effects of three inulin doses on IVTD and neutral detergent fibre digestibility of CS, VO, and PR.

Materials and Methods

This study was conducted in the ruminant feed evaluation laboratory, Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine of Ondokuz Mayis University, Samsun, Turkey. It was approved by the Local Ethics Committee on Animal Experiments of Ondokuz Mayis University (HADYEK 70/2012, 2012.12.18).

Feed samples were obtained from private farms in the province of Samsun in northern Turkey. Dry matter, crude protein, ash, neutral detergent fibre, acid detergent fibre and acid detergent lignin of CS, VO and PR, which are commonly consumed as roughage by ruminants, were determined. Corn crop was harvested at the milk stage (1/2 kernel milkline). A pressed bag silo was opened after two months. To determine dry matter content, CS was oven dried to a constant weight at 65 °C, ground in a mill to pass a 1-mm mesh screen, and those of VO and PR were determined by drying at 105 °C overnight, followed by equilibration in a desiccator. Organic matter content was calculated as weight loss after combustion at 550 °C for at least four hours. Crude protein was determined with the Kjeldahl method. Crude protein was calculated by multiplying the percentage of nitrogen by 6.25 factor. Fat content was determined with ether extraction in the Soxhlet extraction system (Extraction system B-811, Switzerland) (AOAC, 2006). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) levels of each feed were determined with the ANKOM 200 Fiber Analyzer, using methods by Van Soest *et al*. (1991). Sodium sulphide and α-amylase were used for the determination of NDF.

The Daisy incubation method involved the digestion of ground plant biomass in buffered rumen fluid for 48 hours in a Daisy incubator (Ankom Technology Corp., Fairport, NY, USA), followed by NDF digestion in an ANKOM 200/220 Fiber Analyzer (Ankom Technology Corp., Fairport, NY, USA). The process was conducted according to the operating instructions supplied by ANKOM. The study was designed to have CS, VO, and PR in each group. Feed samples (0.5 ± 0.05 g per bag) were weighed into bags (F57) and closed with a heat sealer. The entire experiment involved the transfer of 16 bags of each feedstuff into digestion jars in the incubator. Inulin was added to the fermenter at concentrations of 0, 100, 200, and 300 mg/litre of total culture fluid with the *in vitro* Daisy^{II} Incubator. Each fermenter contained 1600 ml buffer solution and 400 ml rumen fluid. So the control group had 0 mg inulin (CSC, VOC, PRC) added to the fermenter and the treatment groups had 200 mg (CS100, VO100, PR100), 400 mg (CS200, VO200, PR200),and 600 mg of inulin (CS300, VO300, PR300), respectively. Karayaka sheep are an indigenous breed of Turkey that are reared along the coastline of the Black Sea. Karayaka sheep are long thin-tailed breeds. Three fistulated Karayaka rams, which were two years old with average 50 kg live weight, were used for IVTD^{DM} method. The animals were fed a diet containing 650 g alfalfa hay (2320 kcal/kg metabolizable energy; 18.9% crude protein) and 350 g concentrate at 08:00 and 17:00 during the study. Concentrate diet (2500 kcal/kg ME;13% CP) was purchased from Samsun Feed Processing Factory. Fresh water was supplied *ad libitum*. After three weeks' feed adaptation, rumen fluid was collected from three ruminally cannulated Karayaka rams before the morning feeding. The rumen fluid was strained through four layers of cheese cloth to separate the liquid and solid fractions, and then kept at 39 °C under a CO_2 atmosphere. Anaerobic conditions were maintained throughout the preparation stages and conduct of the experiment. The CS, VO and PR were used for the 48-hour incubation. The jars were removed from the chamber at the end of the 48-hour incubation period. The incubation solution was discarded and the bags were rinsed four times with distilled water. To determine IVTD, the bags were placed in an ANKOM fibre analyser and boiled in NDF solution for 75 min. The bags were then removed and soaked twice in acetone for 5 min at each soaking and dried at 100 °C for 24 hours. IVTD was calculated as the difference between DM incubated and the residue after neutral detergent treatment (Van Soest *et al*., 1991). *In vitro* true neutral detergent fibre was calculated as the difference between the amount of fibre incubated and the amount recovered after the neutral detergent treatment.

The initial pH value was determined as soon as the rumen fluid was collected from the Karayaka rams. The pH of the total culture fluid (1600 ml buffer solution+400 ml rumen fluid) from each fermenter at 48 hours after incubation was measured immediately with a pH meter (JENCO Instrument, 6230 M model,

USA). After the mixing 0.6 g methyl green, 8 g sodium chloride (NaCl) and 100 ml 37% formaldehyde solution for staining the protozoans, the volume was increased to 1000 ml with distilled water. One millilitre of rumen inoculum from the fermenter was mixed with 1 mL protozoa suspension. Counting the protozoans was carried out with the object slide of a light microscope and Fuchs-Rosenthal counting chamber (depth: 0.2 mm, small square area: 0.0625 mm^2) (Ranilla *et al.*, 1997).

Data on chemical composition, *in vitro* true digestibility and *in vitro* true neutral detergent fibre digestibility by groups were subjected to ANOVA by one-way experimental design within the groups, and Duncan's multiple range test was done to compare the means (SAS, 2013).

Results and Discussion

Nutrients in forages such as silage, hay or fresh grass are fermented with enzymes produced by rumen microorganisms in ruminants. Because NDF and ADF levels of feeds both affect digestibility, they are important parameters of feed quality. Crude protein, ash, ADF, NDF and ADL contents of CS, VO and PR in this study are presented in Table 1. Similar findings for chemical composition were reported for four varieties of PR by Taweel *et al.* (2006). The NDF and ADL contents of PR in the present study were in accordance with the results of Faville *et al.* (2010). The obtained crude protein value of CS was similar to those of Canbolat *et a*l. (2010), and Muglali *et al.* (2012), who reported that the crude protein level ranged from 7.25 to 8.78 %. Percentages of NDF and ADF values of the current study were similar to those reported for CS by Cetinkaya & Erdem (2015) and Canbolat *et al*. (2010).The findings of the current study showed that the ADF and NDF values of VO were similar to those of Yilmaz *et al.* (2015).

Table 1 Mean (±SE) chemical composition of feed ingredient, %

Parameters	CS	VO	PR
DM (as feed)	28.36 ± 0.355	-	19.23 ± 0.335
DM	95.20 ± 0.785	93.01 ± 0.500	94.62 ± 0.080
CF	3.07 ± 0.110	1.64 ± 0.045	4.61 ± 0.060
CP	7.76 ± 0.115	16.75 ± 0.625	21.16 ± 0.720
Ash	5.20 ± 0.035	9.11 ± 0.040	12.07 ± 0.020
ADF	29.52 ± 0.342	34.09 ± 0.249	21.77 ± 0.156
NDF	45.38 ± 1.074	45.38 ± 0.123	39.43 ± 0.399
ADL	4.94 ± 0.206	7.01 ± 0.222	3.18 ± 0.133

DM: dry matter, CF: crude fibre, CP: crude protein; ADF: acid detergent fibre; neutral detergent fibre; ADL: acid detergent lignin; CS: corn silage; VO: vetch/oat hay; PR: perennial ryegrass

The pH values of rumen fluid collected at the beginning and end of the trial from three ruminally cannulated Karayaka rams for *in vitro* true digestibility determination are shown in Table 2. pH values for CS PR and VO ranged from 6.13 to 6.23, 6.43 to 6.56, and 6.33 to 6.39, respectively, at 48 hours. These pH values were within the normal physiological range. The present study showed that inulin did not cause a significant decline in pH values. There was a variation of 0.06 – 0.13 pH units in the rumen fluid taken separately from each feedstuff. These differences were not sufficient to affect the rumen microorganisms, although the pH value decreased with the addition of increasing amounts of inulin. Some rumen bacteria (*Bifidobacteria, Lactobacilli* etc.) use fructans such as inulin (Kamra, 2005), but they did not cause a substantial reduction in the pH values at 48 hours after incubation when increasing amounts of inulin were added. The main reason may be the relatively slow microbial breakdown of inulin in the medium with rumen fluid. Similar results for pH were found by Ozturk (2009).

Maintaining rumen pH is important for the persistence and productivity of protozoa (Franzolin *et al*., 2010). Protozoa counts in 1 ml rumen fluid of CS, VO and PR digestion at the beginning of the study were 706.400, 680.000 and 568.800, respectively. Furthermore, *Entodium* sp*.* was the dominant species in the rumen fluid taken separately from each feedstuff. Dönmez *et al*. (2003) reported that the counts of rumen protozoa in ruminants fed various diets ranged from 380.000 to 1.160.000 per ml rumen fluid. Monteils *et al.* (2012) reported that the rumen protozoa count of bulls fed diets based on CS was 701.000 in 1 ml rumen

fluid. The results of the study for rumen protozoa count were consistent with those of Dönmez *et al*. (2003) and Monteils *et al*. (2012).

Table 2 pH values of rumen fluid at the beginning of the trial and total culture fluid at 48 hours after incubation

Incubation	**CSC***	**CS100***	**CS200***	**CS300***
Initially (0 h)			6.26	
48 h	6.23	6.22	6.18	6.13
Incubation	**VOC***	**VO100***	**VO200***	**VO300***
Initially (0 h)			6.59	
48 h	6.56	6.52	6.48	6.43
Incubation	**PRC***	**PR100***	**PR200***	**PR300***
Initially (0 h)			6.43	
48 h	6.39	6.38	6.36	6.33

*Inulin added at 0 (CSC, PRC, VOC), 100 (CS100, PR100, VO100), 200 (CS200, PR200, VO200), and 300 (CS300, PR300, VO300) mg/litre of total culture fluid; CS: corn silage; VO: vetch/oat hay; PR: perennial ryegrass

Several factors (plant type, climate, season, soil type and fertility, etc.) affect the nutritive value of forages. Improving the digestibility of fibrous feeds may be possible by using feed additives to increase degradation of the cell wall. The IVTD values after adding three amounts of inulin to CS are presented in Table 3. The $IVTD^{DM}$ values for CSC, CS100, CS200, and CS300 were 60.20 ± 1.022, 58.30 ± 1.08, 59.97 ± 0.472, and 59.23 ± 0.338, respectively. For CS, the $IVTD^{DM}$ values were not significantly different (P >0.05) among the control and treatment groups. Deniz *et al.* (2001) reported *in vitro* organic matter digestibility values of CS harvested at different maturities in the range of 69.52 to 79.18%, which were higher than the results of the present study. The main reason for this difference in $IVTD^{OM}$ may be the use of different methods (*in vitro* or enzyme-based techniques etc.). Therefore, results may not reflect the maximum digestibility of CS.

Table 3 Mean (±SE) *in vitro* true digestibility of corn silage incubated in buffered rumen fluid with various levels of inulin

Digestibility	**CSC***	**CS100***	**CS200***	**CS300***	**P**
$IVTD^{as\ feed}$,%	61.95 ± 0.969	60.31 ± 1.029	61.90 ± 0.450	61.1 9± 0.322	0.187 (NS)
$IVTD^{DM}$,%	60.20 ± 1.022	58.30 ± 1.080	59.97 ± 0.472	59.23 ± 0.338	0.153 (NS)
$IVTD^{NDF}$,%	16.85 ± 2.202	12.54 ± 2.267	16.05 ± 0.99	14.48 ± 0.711	0.129 (NS)
$IVTD^{OM}$,%	62.05 ± 0.988	59.99 ± 1.124	61.63 ± 0.511	60.8 9± 0.288	0.128 (NS)

* Inulin added at 0 (CSC) 100 (CS100), 200 (CS200), and 300 (CS300) mg/litre of total culture fluid; CS: corn silage; NS: non significant; $IVTD^{as\ feed}$: *in vitro* true digestibility as fed; $IVTD^{DM}$: *in vitro* true dry matter digestibility; $IVTD^{NDF}$: *in vitro* true neutral detergent fibre digestibility; $IVTD^{OM}$: *in vitro* true organic matter digestibility

The $IVTD^{NDF}$ provides more accurate estimates of total digestible nutrients, net energy, and feed intake potential (NRC, 2001). The IVNDF digestibilities of CSC, CS100, CS200, and CS300 in the present study were 16.85 ± 2.202, 12.54 ± 2.267, 16.05 ± 0.99, and 14.48 ± 0.711%, respectively. There were no significant differences (P >0.05) among the control and treatment groups. The $IVTD^{NDF}$ in the present study was numerically lower for CSC and CS200 than in the other groups (CS100 and CS300). The $IVTD^{NDF}$ is an important parameter for determining forage quality and feed intake and consequently the productivity of

ruminants. The $IVTD^{NDF}$ reported by Spanghero *et al*. (2010) for CS at the end of the 48-hour incubation period was similar to the control group of the present study. Furthermore, Oba & Allen (2016) determined that the *in vitro* true neutral detergent fibre digestibility values of CS ranged from 35.1 to 48.1% at 24 hours. This may be because of the wide range of the NDF content of the CS samples (DM: 37.0 to 48.3%).

The IVTD values after adding three amounts of inulin to VO are given in Table 4. The $IVTD^{DM}$ values for VOC, VO100, VO200, and VO300 were 63.49 ± 0.298, 62.82 ± 0.352, 63.85 ± 1.279, and 64.10 ± 0.521%, respectively. The $IVTD^{DM}$ values showed no significant differences (P >0.05) among the control and treatment groups. The *in vitro* dry matter digestibility levels of common vetch/wheat hay harvested at different times were in the range of 57.37 to 70.48% (Aksoy & Nursoy*,* 2010). The reported $IVTD^{DM}$ values for common vetch were 58.06 % and 74.95% in 2003 and 2004, respectively (Yucel & Ayasan, 2010). Results in the present study were higher than the values of Yucel & Ayasan (2010) in 2003 and lower than in 2004. Mpairwe *et al.* (2003) reported *in vivo* NDF digestibility values for oats/common vetch hay between 65.3 and 68.4%, which were higher than in the present study.

Table 4 Mean (±SE) *in vitro* true digestibility of common vetch/oat hay incubated in buffered rumen fluid with various levels of inulin

Digestibility	VOC*	VO100*	VO200*	VO300*	P
$IVTD^{as\ feed}$,%	66.04 ± 0.277	65.42 ± 0.327	66.38 ± 1.189	66.61 ± 0.485	0.275 (NS)
$IVTD^{DM}$,%	63.49 ± 0.298	62.82 ± 0.352	63.85 ± 1.279	64.10 ± 0.521	0.275 (NS)
$IVTD^{NDF}$,%	25.17 ± 0.612	23.80 ± 0.721	25.91 ± 2.622	26.43 ± 1.068	0.275 (NS)
$IVTD^{OM}$,%	65.65 ± 0.289	65.092 ± 0.427	65.84 ± 1.351	66.27 ± 0.569	0.339 (NS)

* Inulin added at 0 (VOC) 100 (VO100), 200 (VO200), and 300 (VO300) mg/litre of total culture fluid; VO: vetch/oat hay; NS: non significant; $IVTD^{as\ feed}$: *in vitro* true digestibility as fed; $IVTD^{DM}$: *in vitro* true dry matter digestibility; $IVTD^{NDF}$: *in vitro* true neutral detergent fibre digestibility; $IVTD^{OM}$: *in vitro* true organic matter digestibility

The IVTD values after adding inulin to three amounts of PR are presented in Table 5. *In vitro* true digestibility ($IVTD^{DM}$) for PRC, PR100, PR200, and PR300 were 70.06 ± 1.133, 73.21 ± 4.153, 70.36 ± 0.506, and 66.69 ± 1.317%, respectively. The mean $IVTD^{DM}$ and $IVTD^{as\ feed}$ values of the inulin group (PR300) were significantly lower (*P* <0.05) than control and other treatment groups. Zhao *et al.* (2014) reported that inulin treatment reduced the molar proportion of acetate, the acetate:propionate ratio and methane production compared with starch treatment, which was in line with the current findings. These effects were greater with increased rumen degradable protein. The high dose of inulin (PR300) x rumen degradable protein interactions may have suppressed digestibility by affecting the fermentation parameters in the fermenter.

Table 5 Mean (±SE) i*n vitro* true digestibility of perennial ryegrass incubated in buffered rumen fluid with various levels of inulin

Digestibility	PRC*	PR100*	PR200*	PR300*	P
$IVTD^{as\ feed}$, %	71.67^{ab} ± 1.072	74.65^{a} ± 3.933	71.96^{ab} ± 0.479	68.48^{b} ± 1.246	0.0399
$IVTD^{DM}$,%	70.06^{ab} ± 1.133	73.21^{a} ± 4.153	70.36^{ab} ± 0.506	66.69^{b} ± 1.317	0.0399
$IVTD^{NDF}$,%	28.16^{ab} ±2.718	35.70^{a} ± 9.967	28.88^{ab} ± 1.214	20.06^{b} ± 3.159	0.0399
$IVTD^{OM}$,%	72.10^{ab} ±1.038	75.32^{a} ± 4.167	72.34^{ab} ± 0.454	68.55^{b} ± 1.151	0.0352

* Inulin added at 0 (PRC) 100 (PR100), 200 (PR200), and 300 (PR300) mg/litre of total culture fluid; PR: perennial ryegrass; ^{ab} Means within the same rows with different superscripts are significantly different (P <0.05); $IVTD^{as\ feed}$: *in vitro* true digestibility as fed; $IVTD^{DM}$: *in vitro* true dry matter digestibility; $IVTD^{NDF}$: *in vitro* true neutral detergent fibre digestibility; $IVTD^{OM}$: *in vitro* true organic matter digestibility

The $IVTD^{DM}$ and $IVTD^{as\ feed}$ values decreased with the addition of increasing levels of inulin, except for PR100. Beecher *et al.* (2013) stated that the highest and lowest organic matter digestibilities of various PR cultivars were 75.2 and 69.6%, respectively. The findings of the present study for organic matter digestibility in all groups were similar to those of Beecher *et al*. (2013). Jensen *et al.* (2003) reported that $IVTD^{DM}$ and $IVTD^{NDF}$ values for perennial ryegrass were higher than those obtained in the present study. Higher $IVTD^{DM}$ and $IVTD^{NDF}$ concentrations may be due to harvesting the PR earlier or later in the season, and to fertilization and irrigation levels. The degradation of organic matter by rumen microbes is required for microbial protein synthesis and small chain fatty acid production (Thirumalesh & Krishnamoorthy, 2013). Ozturk (2009) reported that organic matter digestibility was not affected by inulin of Chinese and German origin, which is in agreement with the CS and VO results of the present study.

Conclusion

In conclusion, the findings of the present study showed that supplementation of inulin to CS and VO did not have any effect on $IVTD^{DM}$ and $IVTD^{NDF}$ values. However, a high dose addition of inulin to PR300 reduced $IVTD^{DM}$ and $IVTD^{NDF}$, whereas a low dose addition of inulin to PR100 increased $IVTD^{DM}$ and $IVTD^{NDF}$. Consequently, *i*n vivo digestibility studies with PR may be required to show the effects of different levels inulin supplementation to support the *in vitro* true digestibility findings of the current study.

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Authors' Contributions

MS designed the research project and wrote the manuscript. All co-authors participated in results, statistics and interpretation. All authors read and approved the final manuscript.

Conflict of Interest Declaration

Authors declare that there is no conflict of interest for this work.

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