Effects of Replacing Alfalfa Hay with a Combination of Dried Corn Fiber Feed and Chinese Wildrye on In Vitro Rumen Fermentation: Postprint

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Abstract

The present study was conducted to investigate the effects of partially replacing alfalfa hay in dairy cow diets with a combination of dry corn gluten feed (DCGF) and Leymus chinensis on in vitro rumen fermentation. Using a dairy cow diet as the fermentation substrate, combinations of DCGF and Leymus chinensis were proportionally substituted for 0, 5.00%, 10.50%, 17.50%, and 22.90% of alfalfa hay, with DCGF proportions in the substrate being 0, 3.00%, 7.00%, 11.00%, and 15.00%, respectively, designated as the 0DCGF, 3DCGF, 7DCGF, 11DCGF, and 15DCGF groups. In vitro fermentation parameters at 24 h, microbial community composition, and gas production parameters at 48 h were determined. The results demonstrated: 1) The DCGF and Leymus chinensis combination significantly affected in vitro gas production, potential gas production, and gas production rate ($P<0.05$), all of which increased initially and then decreased with increasing replacement level, peaking in the 11DCGF group; the combination also significantly affected in vitro dry matter disappearance rate ($P<0.05$), with the 11DCGF and 15DCGF groups being significantly higher than other groups ($P<0.05$). 2) The combination significantly influenced concentrations of microbial protein, ammonia nitrogen, acetate, propionate, butyrate, and total volatile fatty acids in fermentation fluid ($P<0.05$), all exhibiting a trend of increasing initially and then plateauing with increasing replacement level; the combination also significantly affected fermentation fluid pH ($P<0.05$), which showed a trend of decreasing initially and then plateauing with increasing replacement level. 3) The combination significantly impacted relative abundances of Ruminococcus flavefaciens, Fibrobacter succinogenes, and Butyrivibrio fibrisolvens in fermentation fluid ($P<0.05$), all displaying a trend of increasing initially and then plateauing with increasing replacement level;

relative abundances of Streptococcus bovis, Succinimonas amylolytica, Ruminobacter amylophilus, and Ruminococcus albus in fermentation fluid did not differ significantly among groups (P>0.05). In conclusion, utilizing a combination of DCGF and Leymus chinensis to replace partial alfalfa hay in dairy cow diets is beneficial for in vitro rumen fermentation, with replacement levels of 17.50% and 22.90% alfalfa hay demonstrating superior in vitro fermentation effects, corresponding to DCGF proportions of 11.00% and 15.00% in the diet, respectively.

Full Text

Effects of Replacing Alfalfa Hay with Dry Corn Gluten Feed and Chinese Leymus on in Vitro Rumen Fermentation

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Abstract

This study investigated the effects of replacing alfalfa hay with combinations of dry corn gluten feed (DCGF) and Chinese leymus on in vitro rumen fermentation in dairy cows. Using a dairy cow diet as the fermentation substrate, DCGF and Chinese leymus combinations were used to replace 0, 5.00%, 10.50%, 17.50%, and 22.90% of alfalfa hay in the substrate at equal proportions, with DCGF proportions of 0, 3.00%, 7.00%, 11.00%, and 15.00% in the substrate, designated as 0DCGF, 3DCGF, 7DCGF, 11DCGF, and 15DCGF groups, respectively. Fermentation parameters and microbial flora were measured after 24 h of in vitro fermentation, and gas production parameters were determined after 48 h. The results showed that: (1) Replacing alfalfa hay with DCGF and Chinese leymus significantly affected gas production, potential gas production, and gas production rate (P<0.05), all of which first increased and then decreased with increasing replacement ratio, with the 11DCGF group showing the highest values. The replacement also significantly affected dry matter disappearance rate (P<0.05), with the 11DCGF and 15DCGF groups being significantly higher than other groups (P<0.05). (2) The replacement significantly affected concentrations of microbial protein, ammonia nitrogen, acetic acid, propionic acid, butyric acid, and total volatile fatty acids in fermentation fluid (P<0.05), all showing a trend of initial increase followed by stabilization. The replacement also significantly affected fermentation fluid pH (P<0.05), which showed a trend of initial decrease followed by stabilization. (3) The replacement significantly affected the

relative counts of *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens* in fermentation fluid ($P<0.05$), which showed a trend of initial increase followed by stabilization. No significant differences were observed among groups in relative counts of *Streptococcus bovis*, *Succinimonas amylolytica*, *Ruminobacter amylophilus*, or *Ruminococcus albus* ($P>0.05$). In conclusion, replacing partial alfalfa hay with DCGF and Chinese leymus in dairy cow diets improved in vitro rumen fermentation, with replacement ratios of 17.50% and 22.90% alfalfa hay (corresponding to DCGF proportions of 11.00% and 15.00% in the diet) showing better fermentation effects.

Keywords: dry corn gluten feed; alfalfa hay; gas production; rumen fermentation; microflora

Introduction

China' s livestock industry has developed rapidly, leading to a substantial increase in forage consumption and a growing shortage of feed grains and high-quality forage. The development of China' s dairy industry is constrained by the shortage of high-quality roughage resources domestically. As the livestock industry continues to expand, the supply-demand gap for high-quality forage is increasing at an annual rate of approximately 10%. According to statistics, China' s total alfalfa imports exceeded 1.2 million tons in 2015, a year-on-year increase of 37.2%, making China the world' s largest alfalfa importer [1].

In recent years, China' s corn starch processing industry has developed rapidly, with large quantities of corn used for starch production annually. This process generates substantial byproducts, including corn fiber, corn steep liquor, corn protein feed, and corn germ meal. Corn gluten feed (CGF) is one such byproduct from wet-process corn starch production, composed of corn steep liquor and corn fiber. CGF is characterized by high crude protein content, high fiber content, high solubility, and high bulk density, with a net energy for lactation of 7.99 MJ/kg for dairy cows [2]. CGF is inexpensive, and its rational use in livestock feed is important for reducing production costs and improving economic benefits.

Numerous studies have demonstrated that CGF can be used as an inexpensive feed ingredient for ruminant production without adverse effects on production performance or animal health when used at appropriate ratios [3-4]. In foreign countries, CGF is more commonly utilized as wet corn gluten feed (WCGF), with few studies investigating dry corn gluten feed (DCGF) for dairy cows. Macleod [5] found that replacing 40% soybean meal or 50% corn with WCGF in dairy cow diets had no significant effect on dry matter intake, improved milk quality, and increased milk fat percentage. Montgomery et al. [6] reported that feeds rich in rapidly fermentable carbohydrates may cause rapid fermentation in the rumen, potentially leading to metabolic disorders and subacute ruminal acidosis. However, feeding WCGF can effectively avoid such metabolic disorders because its main energy source is fermentable fiber rather than starch. DCGF retains most of the nutritional characteristics of WCGF and is more convenient

for storage and transportation. Firkins et al. [7] showed that a combination of 20% DCGF and 1% sodium bicarbonate could replace the same proportion of corn silage in dairy cow diets with good feeding results. Currently, research on DCGF as a fiber source to replace alfalfa hay in dairy cow diets is scarce, and relevant data are limited. Due to its small fiber length, DCGF cannot effectively stimulate rumination and must be combined with long-fiber feeds (such as Chinese leymus) to ensure normal rumen fermentation function. This study used an in vitro gas production method to investigate the effects of replacing partial alfalfa hay with DCGF and Chinese leymus combinations on in vitro rumen fermentation, providing data and theoretical basis for DCGF application in dairy production.

Materials and Methods

1.1 Fermentation Substrate Preparation and Composition Analysis

The experiment was conducted at the Alltech IFM Laboratory of the Nestlé Dairy Farming Training Center in Shuangcheng City, Heilongjiang Province. A dairy cow diet served as the fermentation substrate, with concentrate feed processed by Harbin Fubang Feed Company and roughage samples obtained from a comprehensive ranch in Harbin, Heilongjiang Province. Combinations of DCGF and Chinese leymus replaced 0, 5.00%, 10.50%, 17.50%, and 22.90% of alfalfa hay in the substrate at equal proportions, with DCGF proportions of 0, 3.00%, 7.00%, 11.00%, and 15.00% in the substrate, designated as 0DCGF, 3DCGF, 7DCGF, 11DCGF, and 15DCGF groups, respectively. Substrate samples were ground to pass through a 1 mm sieve after preparation. The composition and nutrient levels of the fermented substrates are shown in Table 1 .

1.2 Rumen Fluid Collection and Processing

Two Holstein dairy cows with permanent rumen fistulas, healthy body condition, and milk yield of (24.5±0.8) kg were selected as rumen fluid donors. The cows were housed in free-stall barns, fed at 07:30 and 14:30 daily, and provided water ad libitum. The composition and nutrient levels of the total mixed ration (TMR) for the experimental animals are shown in Table 2 . At 3 h after morning feeding, 1,000 mL of rumen fluid was collected from each cow via rumen fistula, placed in a pre-warmed thermos flask at 39 °C, and quickly transported to the laboratory. The collected rumen fluid was thoroughly mixed and filtered through four layers of cheesecloth while simultaneously infusing carbon dioxide (CO). The entire operation ensured the rumen fluid remained at 39 °C.

1.3 In Vitro Fermentation

The in vitro fermentation system consisted of a fully automatic gas production recording device (Cerabar T PMP131, Memograph M RSG40) produced by Endress+Hauser Company, Germany, with software provided by Alltech. Saturated CO was infused into prepared buffer solution until it became a colorless,

transparent liquid. The buffer solution was preheated to 39 °C and mixed with fresh rumen fluid at a 2:1 (V/V) ratio, maintaining the mixed fermentation liquid in a vortex state at 39 °C with continuous CO infusion.

Samples (0.5 g) were weighed into F75 filter bags of known weight (dried at 105 °C for 2 h before weighing) and sealed. Samples were placed into corresponding numbered fermentation bottles, infused with CO , and sealed. All fermentation bottles were connected to the gas pressure recording system, and pressure changes were recorded continuously for 10-12 h to check bottle airtightness. After confirming good airtightness, 100 mL of mixed fermentation liquid was pumped into each bottle using a vacuum pump. Each sample had 6 replicates, with 3 blanks designed for data correction. Samples were fermented continuously for 24 and 48 h, with real-time monitoring and automatic recording of gas production.

1.4 Sample Collection and Processing

After 24 h of in vitro fermentation, 3 fermentation bottles from each group were removed. Five milliliters of fermentation fluid was collected into centrifuge tubes and stored at -80 °C for total microbial DNA extraction. Filter bags were removed and rinsed under tap water until the rinse water was colorless and odorless, then dried at 105 °C for 4 h to constant weight to calculate dry matter disappearance rate (DMD). An additional 50 mL of fermentation fluid was collected into centrifuge tubes for pH measurement, then centrifuged at 3,500 r/min for 15 min to determine volatile fatty acids (VFA), ammonia nitrogen (NH -N), and microbial protein (MCP) concentrations. Samples for VFA and NH -N analysis were mixed with 25% metaphosphoric acid solution (5:1, V/V) and stored at -20 °C until analysis.

1.5 Measurement Indicators and Methods

Dry matter, crude ash, and crude protein contents of fermentation substrates and TMR, as well as dry matter content of residues after 24 h fermentation, were determined according to AOAC (2000) [9]. Starch content was determined enzymatically using amyloglucosidase and -amylase purchased from Megazyme, Ireland. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using an Ankom 220 fiber analyzer (ANKOM Company, USA) according to Van Soest et al. [10].

Fermentation fluid pH was measured using a Sartorius Basic pH Meter PB-20. NH -N concentration was determined by the indophenol colorimetric method reported by Broderick et al. [11]. VFA concentration was determined using a Shimadzu GC-200 gas chromatograph with the following conditions: injector temperature 220 °C, carrier gas nitrogen, split ratio 40:1, injection volume 0.4 L; column flow 2.0 mL/min, average linear velocity 38 cm/s; oven temperature program: 120 °C for 3 min, then increased to 180 °C at 10 °C/min, held for 1 min; detector parameters: hydrogen flow 40 mL/min, air flow 450 mL/min,

makeup flow 45 mL/min, FID temperature 250 °C.

MCP concentration was determined by the purine base method [12]. A standard curve was prepared using yeast RNA as standard. Ten milliliters of fermentation fluid was processed and UV absorbance was measured at 260 nm using a UV-2000 spectrophotometer. RNA concentration was calculated based on absorbance values and the standard curve. The MCP calculation formula was:

Microbial protein nitrogen concentration (mg/mL) = RNA concentration (mg/mL) × RNA nitrogen content (17.83%) / bacterial RNA nitrogen content (10%) × dilution factor;
MCP concentration (mg/mL) = Microbial protein nitrogen concentration (mg/mL) × 6.25.

For microbial relative quantification, total DNA was extracted from fermentation fluid using the bead-beating CTAB method [13]. DNA concentration and purity were determined by UV spectrophotometry, ensuring the OD /OD ratio was between 1.6-1.8 and agarose gel electrophoresis showed a bright band near the loading well. DNA samples were stored at -20 °C.

Real-time quantitative PCR (RT-qPCR) was used to detect microbial relative counts using an ABI7500 PCR instrument. The 20 L reaction system was established according to SYBR Premix Ex Taq™ reagent. The reaction program was: 95 °C pre-denaturation for 5 min, 35 cycles of 95 °C denaturation for 5 s, 50-55 °C annealing for 30 s, and 72 °C extension for 40 s, with 3 replicates per sample. Primer sequences are shown in Table 3 , synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Target bacterial relative counts were expressed as percentages relative to total rumen bacterial 16S rDNA:
Target bacterial relative count (%) = [2^-(Ct_target - Ct_total)] × 100,
where Ct_target is the threshold cycle of the target bacteria and Ct_total is the threshold cycle of total bacteria.

1.6 Gas Production and Parameter Calculation

Gas production at 4, 8, 12, 24, 36, and 48 h was calculated using the dynamic fermentation model:
GP = a + b(1 - e^(-ct)) [19],
where a is gas production from rapidly fermented fraction (mL), b is gas production from slowly fermented fraction (mL), c is gas production rate (%/h), $a+b$ is potential gas production (mL), and GP is gas production at time t (mL). Values for a, b, and c were determined using the principle of nonlinear least squares.

1.7 Data Processing and Statistics

Data were processed using SAS 9.2 software and analyzed statistically using a Mixed model. $P<0.05$ indicated significant differences.

Results

2.1 Effects of Replacing Alfalfa Hay with DCGF and Chinese Leymus on In Vitro Gas Production and Parameters

As shown in Table 4 , gas production at all time points increased with increasing replacement ratio, but the 15DCGF group had significantly lower gas production than the 11DCGF group after 12 h ($P<0.05$), with no significant difference from the 7DCGF group ($P>0.05$). The 11DCGF group had significantly higher gas production than all other groups after 12 h ($P<0.05$). The 7DCGF and 15DCGF groups showed no significant differences in gas production at any time point ($P>0.05$), but both were higher than the 0DCGF group ($P<0.05$). The 0DCGF and 3DCGF groups showed no significant differences in gas production during the first 36 h ($P>0.05$).

The 3DCGF and 7DCGF groups had lower gas production from rapidly fermented fractions, significantly lower than the 11DCGF and 15DCGF groups ($P<0.05$). The 11DCGF group had the highest gas production from slowly fermented fractions and potential gas production, significantly higher than all other groups ($P<0.05$). The 0DCGF group had significantly lower potential gas production than other groups ($P<0.05$) and significantly lower gas production rate than the 7DCGF, 11DCGF, and 15DCGF groups ($P<0.05$).

2.2 Effects of Replacing Alfalfa Hay with DCGF and Chinese Leymus on In Vitro Fermentation Parameters

As shown in Table 5 , the 11DCGF and 15DCGF groups had significantly higher dry matter disappearance rates compared with the 0DCGF group ($P<0.05$). Fermentation fluid pH decreased with increasing replacement ratio, with the 7DCGF, 11DCGF, and 15DCGF groups significantly lower than the 0DCGF and 3DCGF groups ($P<0.05$), and the 11DCGF and 15DCGF groups significantly lower than the 7DCGF group ($P<0.05$). The 11DCGF and 15DCGF groups had higher NH -N concentrations, while the 0DCGF and 3DCGF groups had lower concentrations, with significant differences between them ($P<0.05$). Partial replacement of alfalfa hay increased fermentation fluid MCP concentration, with the 7DCGF and 11DCGF groups significantly higher than the 0DCGF group ($P<0.05$).

Partial replacement of alfalfa hay significantly increased total volatile fatty acid concentration ($P<0.05$), with the 11DCGF and 15DCGF groups showing higher values. The 11DCGF and 15DCGF groups had significantly higher acetic, propionic, and butyric acid concentrations than the 0DCGF group ($P<0.05$). The acetate/propionate ratio decreased with increasing replacement ratio, with the 0DCGF group significantly higher than all other groups ($P<0.05$).

2.3 Effects of Replacing Alfalfa Hay with DCGF and Chinese Leymus on In Vitro Fermentation Fluid Microflora

As shown in Table 6 , for fiber-degrading bacteria: the 11DCGF and 15DCGF groups had significantly higher relative counts of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* than the 0DCGF and 3DCGF groups ($P<0.05$). The 7DCGF, 11DCGF, and 15DCGF groups had significantly higher relative counts of *Butyrivibrio fibrisolvens* than the other two groups ($P<0.05$), with the 11DCGF and 15DCGF groups significantly higher than the 7DCGF group ($P<0.05$). No significant differences were observed among groups in relative counts of *Ruminococcus albus* ($P>0.05$). For starch-degrading bacteria: no significant differences were found among groups in relative counts of *Streptococcus bovis*, *Ruminobacter amylophilus*, or *Succinimonas amylolytica* ($P>0.05$).

Discussion

Gas production from dietary fermentation by rumen microorganisms is an important indicator of feed fermentability, positively correlated with nutrient digestibility [20]. Dietary carbohydrates and crude protein are the main sources of fermentation gas, and fermentable nutrient content and rumen microbial activity are significantly correlated with gas production [21]. In this study, the 11DCGF group had higher gas production at all time points and higher gas production from slowly fermented fractions than other groups, indicating the highest diet digestibility and highest content of fermentable organic matter. DCGF contains high levels of degradable crude protein and fermentable fiber, with a greater degradable portion in the rumen than other roughages [4]. Silva et al. [22] suggested that different roughage combinations may produce positive associative effects, improving the digestibility of low-quality roughage. When low-quality and high-quality roughages are combined, dominant fiber-degrading microbial populations first attach to easily digestible fibers, proliferate rapidly, and thereby increase the degradation rate of low-quality roughage, subsequently increasing gas production and concentrations of NH -N and VFA. Therefore, partial replacement of alfalfa hay with DCGF and Chinese leymus in this study may have produced positive associative effects, improving the degradation rate of less digestible Chinese leymus and consequently increasing gas production in the 7DCGF, 11DCGF, and 15DCGF groups compared with the 0DCGF and 3DCGF groups. The 0DCGF diet had relatively simple roughage composition; alfalfa hay has high dry matter digestibility, higher ADF content than DCGF, and lower NDF content, resulting in relatively higher gas production from rapidly fermented fractions but lower gas production from slowly fermented fractions and lower gas production rate.

pH is an important indicator reflecting the rumen internal environment and a key factor affecting microbial growth and metabolism. pH deviating from the normal range reduces rumen microbial activity and is unfavorable for microbial fermentation and growth [23]. The optimal pH range for fiber-degrading bacteria activity is 6.2-6.8; if pH falls below 6.2, fiber-degrading bacterial activity

decreases, reproduction is limited, and fiber degradation rate declines [24]. In this study, fermentation fluid pH showed a decreasing trend with increasing replacement ratio after 24 h fermentation, but remained within the normal range, indicating a stable fermentation environment conducive to microbial growth and metabolism. VFA production and accumulation caused the decrease in rumen fluid pH, which showed a decreasing trend as VFA concentration increased with replacement ratio.

NH -N is the main product of nitrogenous substance degradation in the rumen, and its concentration reflects dietary protein degradation and microbial utilization efficiency. Appropriate NH -N concentration is necessary for efficient MCP synthesis; excessively high concentration indicates mismatched ammonia production and utilization rates, potentially wasting protein, while excessively low concentration inhibits microbial growth and reduces MCP synthesis efficiency [25]. Hoover [26] reported that the optimal ammonia concentration for microbial growth is 3.3-8.0 mg/dL, though rumen NH -N concentration typically fluctuates within a wide range (1-76 mg/dL). In this study, NH -N concentration in fermentation fluid increased with replacement ratio, indicating improved dietary crude protein degradation and providing more substrates for rumen microbial fermentation. DCGF has significantly higher rapidly fermentable crude protein content than alfalfa hay, with higher rumen degradability [27]. Therefore, the 11DCGF and 15DCGF diets had increased degradable crude protein content, providing adequate nitrogen sources for rumen microorganisms. Ruminants primarily utilize energy in the form of VFA, with carbohydrates producing large amounts of VFA during rumen fermentation, of which acetic, propionic, and butyric acids account for over 95% of total volatile fatty acids [28]. Leng et al. [29] suggested that VFA concentration and proportions mainly relate to dietary non-fibrous carbohydrate (NFC) and NDF contents, while roughage quality also affects rumen fermentation patterns and function [30]. In this study, replacing alfalfa hay with DCGF and Chinese leymus combinations increased acetic, propionic, and butyric acid concentrations, possibly because roughage combinations affected rumen microflora and fiber-degrading enzyme activity. Additionally, DCGF is rich in hemicellulose [28]; as DCGF proportion increased, dietary hemicellulose content increased while ADF content decreased, and increased degradable fiber content raised fermentation fluid VFA concentration, providing sufficient energy for microbial growth and reproduction. Furthermore, higher NFC content can increase dietary degradation rate and extent [31], contributing to higher dry matter disappearance rates in the 11DCGF and 15DCGF groups.

Zhang et al. [32] found that different roughage combinations affect populations of fiber-degrading bacteria such as *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens*, thereby affecting dietary fiber degradation and VFA concentration. According to Silva et al.' s [22] associative effects theory, rumen microorganisms in this study may have preferentially utilized the more digestible fiber in DCGF, increasing microbial activity and further improving the fiber degradation rate of other roughages with higher ADF content

such as Chinese leymus, thus increasing acetic acid concentration. Different feed types can compensate for nutritional imbalances in single feeds through complementary nutrient interactions, providing better conditions for rumen microbial metabolism, improving rumen fermentation level and MCP concentration, and supplying more nutrients for dairy production [33]. In this study, partial replacement of alfalfa hay with DCGF and Chinese leymus improved overall fermentation level, increased NH -N and VFA concentrations, accelerated microbial growth and metabolism, and increased MCP concentration, possibly due to positive associative effects among DCGF, Chinese leymus, alfalfa hay, and corn silage, as indicated by in vitro gas production parameters.

Major fiber-degrading bacteria in the rumen include *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens*. This study showed that relative counts of *R. flavefaciens*, *F. succinogenes*, and *B. fibrisolvens* in fermentation fluid increased with replacement ratio after 24 h fermentation. Silva et al. [22] suggested that rumen bacteria attach more strongly to easily digestible fiber surfaces than to poorly digestible fibers like straw, facilitating better interaction with digesta and improving microbial reproduction efficiency and feed utilization. *B. fibrisolvens* can degrade both fiber and protein, and the high soluble protein content in DCGF provided adequate nitrogen for its growth.

Conclusion

1. Replacing partial alfalfa hay with DCGF and Chinese leymus in dairy cow diets increased 48 h gas production, potential gas production, and gas production rate, increased 24 h fermentation fluid MCP, NH -N, acetic acid, propionic acid, butyric acid, and TVFA concentrations, and increased relative counts of major fiber-degrading bacteria in 24 h fermentation fluid.
2. Replacement ratios of 17.50% and 22.90% alfalfa hay (corresponding to DCGF proportions of 11.00% and 15.00% in the diet) were appropriate. However, DCGF is a short-fiber feed lacking physically effective fiber, and further in vivo validation is required.

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Note: Figure translations are in progress. See original paper for figures.