

Inter-laboratory Reproducibility of Digestive Conditions and Energy Value Determination of Enzymatic Hydrolysates in a Biomimetic Digestive System for Monogastric Animals: A Postprint

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Abstract

This experiment aimed to investigate the reproducibility of digestion conditions and measurement results of the single-stomach animal biomimetic digestion system (SDS-2) among different laboratories. A single-factor completely randomized design was adopted, with a total of 4 laboratory treatments. Corn and soybean meal were ground to pass through a 60-mesh sieve and then distributed to 4 laboratories. Each laboratory selected one SDS-2 unit, and each SDS-2 unit was set up with 2 biomimetic digestion groups to determine their buffer flow rate, digestive fluid flow rate, and cleaning fluid flow rate. Temperature sensors were used to measure the temperature variation curves of the enzymatic reaction chamber, buffer temperature control chamber, and digestive fluid storage chamber, respectively, while electromagnetic sensors were used to measure the variation curve of mixing frequency. According to the SDS-2 operating technical procedures, the enzymatic hydrolysate energy values of corn and soybean meal were determined. The results showed that: 1) There were significant differences in the small intestine and large intestine digestive fluid flow rates among different laboratories ($P < 0.05$), but no significant differences in the stomach, small intestine, and large intestine buffer flow rates and cleaning fluid flow rates ($P > 0.05$). Within each laboratory, there were significant differences in the stomach, small intestine, and large intestine buffer flow rates and cleaning fluid flow rates between the 2 biomimetic digestion groups of the same SDS-2 unit ($P < 0.05$). 2) In the 4 laboratories, although the initial mixing frequency of the biomimetic digestion system and the initial temperatures of the enzymatic reaction chamber, buffer temperature control chamber, and digestive fluid storage chamber differed after startup, the digestion conditions all reached consistency after 60 min of

operation. 3) The within-laboratory, between-laboratory, and total coefficients of variation for corn dry matter digestibility and enzymatic hydrolysate energy value were all no more than 1.23%, with no significant differences among laboratories ($P>0.05$). The within-laboratory, between-laboratory, and total coefficients of variation for soybean meal dry matter digestibility were 1.30%, 2.23%, and 2.52%, respectively, and the corresponding coefficients of variation for enzymatic hydrolysate energy value were 0.89%, 1.43%, and 1.64%, respectively, with significant differences among laboratories ($P<0.01$). Thus, the differences in SDS-2 small intestine and large intestine fluid flow rates among laboratories did not lead to differences in the measurement results of feed enzymatic hydrolysate energy values. Each laboratory's SDS-2 unit could achieve consistent digestion conditions after operating for more than 60 min. The reproducibility of corn enzymatic hydrolysate energy value determination among laboratories was higher than that of soybean meal; the total laboratory coefficient of variation for the enzymatic hydrolysate energy values of both feedstuffs could be controlled within 1.64%, demonstrating satisfactory reproducibility that meets the requirements for quantitative analysis.

Full Text

Reproducibility of Digestion Condition and Enzymatic Hydrolysate Gross Energy Determination in Simulated Digestion System for Monogastric Animals Across Different Laboratories

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Abstract

This experiment was conducted to investigate the reproducibility of digestion conditions and measurement results of the Simulated Digestion System for monogastric animals (SDS-2) across different laboratories. A single-factor completely randomized design was employed with four laboratory treatments. Corn and soybean meal were ground to pass through a 60-mesh sieve and distributed to four laboratories. Each laboratory utilized one SDS-2 instrument, with each SDS-2 configured with two simulated digestion groups to determine the flow velocities of buffer solution, digestive fluid, and washing solution. Temperature variation curves of the enzymatic reaction chamber, buffer solution temperature control chamber, and digestive fluid storage chamber were measured using temperature sensors, while mixing frequency variation curves were measured using electromagnetic sensors. According to the SDS-2 operating technical specifications, the enzymatic hydrolysate gross energy (EHGE) of corn and soybean meal was

determined. The results showed that: 1) Significant differences were observed in small intestinal and large intestinal digestive fluid flow velocities among different laboratories ($P < 0.05$), but no significant differences were found in gastric, small intestinal, and large intestinal buffer solution flow velocities or washing solution flow velocities among laboratories ($P > 0.05$). Within the same SDS-2 instrument, significant differences existed between the two simulated digestion groups in gastric, small intestinal, and large intestinal buffer solution flow velocities and washing solution flow velocities ($P < 0.05$). 2) Although the initial mixing frequency and initial temperatures of the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage chamber differed among the four laboratories after startup, the digestion conditions became consistent after 60 minutes of operation. 3) For corn, the intra-laboratory, inter-laboratory, and total coefficients of variation (CV) for dry matter digestibility (DMD) and EHGE did not exceed 1.23%, with no significant differences among laboratories ($P > 0.05$). For soybean meal, the intra-laboratory, inter-laboratory, and total CVs for DMD were 1.30%, 2.23%, and 2.52%, respectively, while the corresponding CVs for EHGE were 0.89%, 1.43%, and 1.64%, with significant differences observed among laboratories ($P < 0.01$). In conclusion, differences in small intestinal and large intestinal fluid flow velocities among laboratories did not cause differences in feed EHGE determination results. Consistent digestion conditions could be achieved after operating the SDS-2 for more than 60 minutes. The reproducibility of corn EHGE determination among laboratories was superior to that of soybean meal, and the total CV for EHGE of both feedstuffs could be controlled within 1.64%, demonstrating satisfactory reproducibility that meets quantitative analysis requirements.

Keywords: simulated digestion; digestion condition; enzymatic hydrolysate gross energy; reproducibility

Introduction

National standard GB/T 6379.1–2004 specifies that the accuracy of measurement methods and results includes two aspects: repeatability and reproducibility [1]. Repeatability refers to precision under conditions where independent tests are conducted in the same laboratory by the same operator using the same equipment and test method, while reproducibility refers to precision under conditions where independent tests are conducted in different laboratories by different operators using different equipment according to the same method. These are important criteria for determining whether a method is feasible. In the European method for determining metabolizable energy values in broiler diets established by Bourdillon et al. [2], the average coefficient of variation for nitrogen-corrected apparent metabolizable energy (AMEn) values of four diets among seven laboratories was 2.92%. Carabaño et al. [3] used the method of Boisen et al. [4] to simulate rabbit digestion *in vitro* and obtained an average coefficient of variation for dry matter digestibility (DMD) of eight samples among four laboratories of 3.24%. These results indicate relatively large reproducibil-

ity variation in determining feed nutrient biological values among laboratories. Regarding the repeatability of SDS-2 test results developed by the State Key Laboratory of Animal Nutrition, Li et al. [5] used the first-generation Simulated Digestion System for monogastric animals (SDS-1) to determine duck feed EHGE in the same laboratory, with intra-batch and inter-batch coefficients of variation not exceeding 1.40%. Zhao et al. [6] used the second-generation SDS-2 to determine EHGE of four chicken feed ingredients, with intra-batch and inter-batch coefficients of variation not higher than 1.64%. These results demonstrate satisfactory repeatability of SDS-2. However, environmental conditions differ among laboratories, and the reproducibility of test results requires further verification. Therefore, this study compared digestion conditions including buffer solution flow velocity, digestive fluid flow velocity, washing solution flow velocity, and post-startup temperature and mixing frequency variation curves during SDS-2 simulation of pig digestion, as well as the reproducibility of EHGE determination among four laboratories, to investigate whether the reproducibility performance of SDS-2 across different laboratories meets quantitative analysis requirements.

1.1 Feed Ingredients

Approximately 2.5 kg each of corn and soybean meal were collected, ground using a universal grinder, and passed through a 60-mesh square sieve. Samples were packaged under vacuum with nitrogen filling and protected from light, transported under normal conditions to each laboratory, and stored at -20 °C for later use. The proximate nutrients and gross energy values of corn and soybean meal are presented in Table 1 .

1.2 Experimental Design

All SDS-2 instruments in the laboratories were configured according to the digestion parameters for growing pigs [9] and maintained consistent. Based on the design principle of SDS-2 [7-8], a nested design was adopted to examine differences in buffer solution flow velocity, washing solution flow velocity, and digestive fluid flow velocity between the two simulated digestion pipelines within instruments across laboratories. The primary treatment factor was laboratory (four levels), with one SDS-2 instrument per laboratory. The secondary treatment factor was the two simulated digestion groups within each SDS-2, with three replicate measurements per treatment. Temperatures of the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage chamber were detected by temperature sensors, and mixing frequency was measured by electromagnetic sensors. Differences in DMD and EHGE of feeds among laboratories were examined using a completely randomized design with five replicates per treatment and one digestion tube per replicate.

1.3 Determination of Digestion Conditions in Simulated Digestion

Buffer Solution Flow Velocity Determination: When the rotation speed of peristaltic pump No. 1 in SDS-2 was set to 60 r/min, the volume of deionized water pumped into the first and second simulated digestion group pipelines was measured using a graduated cylinder after 2 minutes of pumping, with three replicate measurements per group.

Washing Solution Flow Velocity Determination: When the rotation speed of peristaltic pump No. 2 in SDS-2 was set to 180 r/min, the volume of deionized water pumped into the first and second simulated digestion group pipelines was measured using a graduated cylinder after 2 minutes of pumping, with three replicate measurements per group.

Digestive Fluid Flow Velocity Determination: 1) For simulated small intestinal fluid injection flow velocity, 2 mL of deionized water was added to tubes No. 1-10 using a pipette, peristaltic pump No. 3 was activated, and the time required for complete pumping into the simulated digester was recorded using a stopwatch, with three replicate measurements. 2) For simulated large intestinal fluid injection flow velocity, 2 mL of deionized water was added to tubes No. 11-20 using a pipette, peristaltic pump No. 4 was activated, and the time required for complete pumping into the simulated digester was recorded using a stopwatch, with three replicate measurements.

Temperature and Mixing Frequency Monitoring: Temperature variation curves of the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage chamber during simulated digestion, as well as mixing frequency variation curves of the shaking table, were collected by temperature sensors and electromagnetic sensors through the SDS-2 control software.

1.4 Determination of Feed EHGE

The pig simulated digestive fluid kit was provided by the State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, transported under low temperature, and stored at 4 °C in each laboratory for later use. The digestive enzyme activities in simulated digestive fluid and the method for determining feed DMD and EHGE by simulated digestion were referenced from the *Manual of Simulated Digestion System for Monogastric Animals* (2nd edition) [9].

1.5 Data Processing

Based on the principle of single-factor completely randomized design, the MEANS module of SAS 9.0 was used to analyze basic statistics. According to the principle of nested design, the ANOVA module was used for variance analysis of flow velocities. The GLM module was used for variance analysis of DMD and EHGE of feed ingredients under each treatment, with means compared using Duncan's multiple comparison method. Intra-laboratory,

inter-laboratory, and total coefficients of variation were calculated according to the method of Jiang and Xia [10]. Data calculation formulas and statistical models are as follows:

The nested design statistical model is:

The single-factor ANOVA statistical model is: $Y_{ijk} = \mu + L_i + B(L)_{ij} + \varepsilon_{ijk}$.

Where: M_1 is the dry matter weight of the sample; M_2 is the dry matter weight of the undigested residue; E_1 is the total energy of the sample; E_2 is the total energy of the undigested residue; \bar{Y}_i is the mean of the i th laboratory; L is the laboratory effect; B is the effect of the two simulated digestion groups within SDS-2 in each laboratory; ε is the error; \bar{Y} is the overall sample mean; G is the number of laboratories; N is the total number of samples; n_i is the number of samples in the i th laboratory.

2.1 Reproducibility of SDS-2 Digestion Condition Measurements Among Different Laboratories

As shown in Table 2, in the determination of SDS-2 control parameters, no significant differences were observed among laboratories in gastric, small intestinal, and large intestinal buffer solution flow velocities or washing solution flow velocities ($P > 0.05$). However, significant differences were found between the two simulated digestion groups within the same instrument for gastric, small intestinal, and large intestinal buffer solution flow velocities and washing solution flow velocities ($P < 0.01$). Specifically, laboratories 2, 3, and 4 showed significant differences in gastric buffer solution flow velocities between their two simulated digestion groups ($P < 0.05$), with differences of 16-18 mL/min. Laboratories 3 and 4 exhibited significant differences in small intestinal and large intestinal buffer solution flow velocities between their two groups ($P < 0.05$), with differences of 12-18 mL/min. Laboratories 1, 2, and 4 showed significant differences in washing solution flow velocities between their two groups ($P < 0.05$), with differences of 3-35 mL/min. Significant differences in small intestinal and large intestinal digestive fluid flow velocities were observed among laboratories ($P < 0.05$), with laboratories 1 and 4 having significantly higher flow velocities than laboratories 2 and 3 ($P < 0.05$). Significant differences in small intestinal digestive fluid flow velocity were found between the two simulated digestion groups ($P < 0.05$), while no significant differences were observed in large intestinal digestive fluid flow velocity between groups ($P > 0.05$).

The variation curves of mixing frequency and temperatures of the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage chamber after SDS-2 startup in the four laboratories are shown in Figure 1 [Figure 1: see original paper]. As shown in Figure 1-a, laboratory 3 had the lowest initial mixing frequency (79 r/min), laboratory 1 had the highest (135 r/min), and laboratories 2 and 4 were intermediate (averaging 108 r/min). After 6 minutes of operation, the mixing frequency in all four laboratories reached the preset value of 180 r/min and maintained this frequency. As shown in Figure 1-

b, laboratory 3 had the lowest initial temperature in the buffer solution control chamber (20.9 °C), laboratory 1 had the highest (39.1 °C), and laboratories 2 and 4 were intermediate (28.0-29.1 °C). After 60 minutes of operation, the buffer solution control chamber temperatures in all four laboratories approached the set value (39.0 °C). As shown in Figure 1-c, laboratory 3 had the lowest initial temperature in the enzymatic reaction chamber (19.0 °C), laboratory 1 had the highest (38.1 °C), and laboratories 2 and 4 were intermediate (28.8-29.8 °C). The temperatures in the enzymatic reaction chambers of all four laboratories approached the set value (39.0 °C) after 24-36 minutes of operation. In laboratory 3, the temperature dropped sharply from 39.0 °C to 31.0 °C at 42-48 minutes after startup due to opening the operation door of the enzymatic reaction chamber, but approached the set value again after 60 minutes when the door was closed. As shown in Figure 1-d, laboratory 1 had the lowest initial temperature in the digestive fluid storage chamber (8.9 °C), laboratories 2 and 4 had the highest (28.2-29.6 °C), and laboratory 3 was intermediate (17.9 °C). After 12 minutes of operation, the digestive fluid storage chamber temperatures in all four laboratories varied within 3.9-11.4 °C.

2.2 Reproducibility of SDS-2 in Determining Feed DMD and EHGE Among Laboratories

As shown in Table 3, in the simulated digestion of corn, no significant differences were observed among the four laboratories for DMD and EHGE ($P > 0.05$), ranging from 78.03% to 78.69% and 15.43 to 15.63 MJ/kg, respectively. The intra-laboratory, inter-laboratory, and total coefficients of variation for corn DMD and EHGE were all below 1.23% and 0.99%, respectively.

In the simulated digestion of soybean meal, significant differences were observed among the four laboratories for both DMD and EHGE ($P < 0.01$), ranging from 58.36% to 61.58% and 13.14 to 13.62 MJ/kg, respectively. The intra-laboratory, inter-laboratory, and total coefficients of variation for soybean meal DMD were 1.30%, 2.23%, and 2.52%, respectively, while the corresponding values for EHGE were 0.89%, 1.43%, and 1.64%.

Discussion

3.1 Control and Variation Factors of SDS-2 Digestion Conditions

In vitro simulated digestion, digestion conditions directly affect the degree of simulated digestion and its correlation with in vivo digestion [11]. However, in traditional simulated digestion using Erlenmeyer flasks as reactors, different researchers have not standardized the settings of in vitro digestion conditions for the same animal species [12]. Even for highly automated in vitro digestion systems, few reports exist on whether the set digestion conditions match the actual conditions [13]. In this experiment, all laboratories used consistent parameter settings for SDS-2 digestion conditions, but the degree to which the measured digestion conditions matched the set parameters was influenced by the control

precision of the electrical components involved in the control process. According to the definition of reproducibility in national standard GB/T 6379.1–2004 [1], under the premise of consistent set digestion condition parameters, SDS-2 instruments in different laboratories still exhibited variation in measured digestion conditions, thereby affecting the degree of reproducibility. From the design principle of SDS-2, within the same instrument, the pumping of buffer solution, simulated small intestinal fluid, and simulated large intestinal fluid into the simulated digester is powered by peristaltic pumps No. 1, 3, and 4, respectively, while the deionized water required for each cleaning is quantitatively pumped by peristaltic pump No. 2 [7] (Table 4). The two simulated digestion groups are powered by coaxially synchronized dual pump heads for solution pumping; therefore, pulse flow is mainly affected by the degree of pump tube wear and pipeline resistance. Preliminary results from our research group showed that when new pump tubes were installed in the pump head (6 rollers) and operated at 60 r/min for 180 hours, the flow velocity decreased from 160 mL/min to 140 mL/min and stabilized. This phenomenon is related to the principle that peristaltic pumps provide pumping power through pulsed extrusion of pump tubes by pump head rollers. New pump tubes have larger extrusion space and thus relatively larger pulse flow; after the pump tubes are extruded by the pump head for a period, the space becomes smaller and stabilizes, so the pulse flow correspondingly becomes smaller and stabilizes. Additionally, the gastric, small intestinal, and large intestinal buffer solutions entering the simulated digester circulation are controlled by three groups (two per group) of solenoid valves, and the length of pipelines and solenoid valve contact extrusion affect liquid flow resistance. In this experiment, buffer solution flow velocities varied between 130-156 mL/min, with no significant differences in average buffer solution flow velocity among laboratories but significant differences between the two simulated digestion groups within the same instrument. This indicates that SDS-2 instruments were generally consistent in buffer solution flow velocity among laboratories but exhibited differences between the two groups within the same instrument. From the principle of monogastric animal simulated digestion, feed and digestive fluid are inside the dialysis bag, while buffer solution is outside the dialysis bag and circulated by the peristaltic pump. When the buffer solution circulation speed greatly exceeds the material exchange speed across the dialysis bag, differences in buffer solution flow velocity will not cause differences in the removal of substances from the dialysis bag or the degree of inhibition of simulated digestion by digestion products. Moreover, since the ratio of sample amount to buffer solution is 5-10 g:1,000 mL, differences in buffer solution flow velocity will not cause differences in hydrolysis product concentration in the buffer solution. In this SDS-2 experiment, buffer solution circulated once every 7-8 minutes, and the difference in buffer solution flow velocity between the two simulated digestion groups required approximately 50 minutes to cause a one-cycle difference in buffer solution circulation, while the material exchange speed across the dialysis bag was about 40 minutes. Therefore, under the conditions of this experiment, differences in buffer solution flow velocity would not cause differences in digestibility. Significant differences in digestive fluid flow

velocity existed among laboratories. According to the principle of monogastric animal simulated digestion, each simulated digester was pumped with 2 mL of simulated small intestinal fluid and simulated large intestinal fluid. To ensure complete pumping of all digestive fluid into the simulated digester, the lowest flow velocity among the 10 channels of peristaltic pumps (No. 3 and 4) was used as the digestive fluid pumping speed, thereby ensuring that 2 mL of digestive fluid could be completely pumped into the simulated digester and eliminating the influence of flow velocity variation on simulated digestion. After simulated digestion, the volume of deionized water used to wash hydrolysis products had a ratio of 300 mL:1-2 g to the sample amount, with four cumulative washes. If the washing solution flow velocity differed by 40 mL/min between the two simulated digestion groups in the same SDS-2 instrument, the volume difference would be within 150 mL after pumping 1,500 mL of deionized water, equivalent to a 30 mL difference per digestion tube. Based on a sample amount of 2 g per digestion tube and a DMD of 75%, the total amount of substances removed by washing solution was 1.5 g. Due to stepwise washing, the theoretical difference in undigested substances between the two simulated digestion groups was: $1.5 \times (20/350)^4 - 1.5 \times (20/320)^4 < 0.0001$ g. Thus, when the total washing solution volume is much larger than the sample amount, after four stepwise washes, even if the pumped washing solution volume differs by 10% each time, the difference in undigested substances can be neglected. The experimental results of Li et al. [5] demonstrated no significant differences in digestibility of the same sample between the two simulated digestion groups within the same SDS-2, indicating that solution flow velocity variation under current SDS-2 design and electrical component conditions does not cause differences in EHGE determination values.

The initial mixing frequency of SDS-2 differed among laboratories due to differences in the actual capacitance of the motors, resulting in different motor speeds upon power-on. After 6 minutes of operation, the mixing frequency in each laboratory reached the set requirement. The initial temperatures of the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage chamber differed significantly among laboratories. Except for laboratory 1, where SDS-2 had just completed one digestion cycle before immediately starting this experiment, the other three laboratories conducted this experiment with SDS-2 that had not been previously started. Therefore, laboratory 1 had temperatures in the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage chamber that were very close to the set parameters, while the other three laboratories had initial temperature conditions close to their respective laboratory ambient temperatures. The buffer solution control chamber required longer time to reach the set temperature than the enzymatic reaction chamber and digestive fluid storage chamber because the heat capacity of water is greater than that of air. Although ambient temperatures differed significantly among laboratories, the temperature conditions all reached the set requirements after 60 minutes of operation. Therefore, setting the SDS-2 warm-up time to 60 minutes can eliminate the influence of inter-laboratory ambient

temperature differences on simulated digestion.

3.2 Reproducibility of SDS-2 in Determining Feed Ingredient DMD and EHGE

In simulated digestion methods, although simulated digestion parameters and process control are consistent among different laboratories and human operation-induced interference is minimized to the greatest extent, differences still exist among laboratories in aspects such as test water specifications, bomb calorimetry for gross energy determination, and operator proficiency. Under the same laboratory conditions, the experimental results of Zhao et al. [6] showed that the intra-batch, inter-batch, and total coefficients of variation for DMD and EHGE of corn, wheat, and cottonseed meal determined by chicken simulated digestion method did not exceed 1.00%. Carabaño et al. [3] used the Erlenmeyer flask in vitro simulation method to determine rabbit diet DMD in four laboratories, with an intra-laboratory coefficient of variation of 1.73% and an inter-laboratory coefficient of variation of 3.24%. Bourdillon et al. [2] determined the coefficients of variation for dry matter content, gross energy, nitrogen content, and apparent metabolizable energy of chicken diets among seven European laboratories as 1.27%, 1.29%, 4.39%, and 2.92%, respectively. Getachew et al. [14] used the gas production method to determine the coefficient of variation for 24-hour gas production among seven laboratories as 7.88%. In this experiment, the intra-laboratory and inter-laboratory coefficients of variation for corn DMD and EHGE determined by simulated digestion method were both within 1.23%, with no significant differences among laboratories. For soybean meal, the intra-laboratory and inter-laboratory coefficients of variation for DMD and EHGE were within 2.23%, with significant differences among laboratories in DMD and EHGE, and the range and coefficient of variation for DMD were larger than the corresponding values for energy digestibility. This may be related to inconsistencies in the water used for washing products during simulated digestion among laboratories (laboratories typically use two types of test water: commercial bottled purified water and membrane-filtered deionized water). Additionally, in the simulated digestion method, the sample amount for soybean meal was 1 g, while for corn it was 2 g, which theoretically doubled the measurement error for soybean meal compared to corn from a data calculation perspective, thereby increasing inter-laboratory differences. Nevertheless, compared with the aforementioned methods, the simulated digestion method in this experiment demonstrated better reproducibility among laboratories.

Conclusion

1. Although significant differences existed in digestive fluid flow velocity among laboratories, they did not cause differences in feed ingredient EHGE determination results. After operating SDS-2 for 60 minutes, consistent mixing frequency and temperatures in the enzymatic reaction chamber, buffer solution control chamber, and digestive fluid storage

chamber were achieved among the four laboratories.

2. The reproducibility of corn EHGE among laboratories was superior to that of soybean meal. The total coefficient of variation for EHGE of both feedstuffs could be controlled within 1.64%, demonstrating satisfactory reproducibility.

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