Safety and nutritional evaluation of biogas residue left after the production of biogas from wastewater

Baoguo Bian,1 Lvmu Li,1 Xiongyuan Si,2 Bin Li,1 Wenjie Guo,3 Hua Mu,3 Xiaoling Ding,1 Fazhi Xu1

1School of Animal Science and Technology, Anhui Agricultural University, Hefei, Anhui, China
2Central of Biotechnology, Anhui Agricultural University, Hefei, Anhui, China
3Anhui Ruifuxiang Food Co. Ltd., Bozhou, Anhui, China

Abstract

We investigated the safety and nutritional value of biogas residue left after the production of biogas from wastewater. In Exp. 1, ninety-six female mice were selected for acute oral toxicity testing and randomly allocated to 4 treatment groups, which received distilled water (control) or the biogas residue solution at 1 g/mL, 5 g/mL, or 15 g/mL. Activity levels and serum biochemical parameters were measured after 24 hours. In Exp. 2, eighty mice were divided into 2 treatment groups for sub-acute and sub-chronic toxicity testing, which received either a control group diet or the biogas residue diet (20% biogas residue). In Exp. 3, to test the nutritional value of the biogas residue, four pigs were fed either a low-casein corn starch-based diet or a semi-purified diet with biogas residue as the only source of protein, and the apparent and true digestibility of crude protein and amino acids, apparent metabolizable energy, and digestible energy were measured. Group differences in serum parameters and mouse weight gain were not significant 24 hours after biogas residue solution gavage, and the viscera appeared normal. At day 30 of the observation period, changes in serum biochemical parameters were not significant, but the mean spleen index of mice treated with biogas residue was greater (P<0.05) than that of the control group. In this study, biogas residue had no significant adverse effects on the body and it was safe as a feed supplement at a 20% replacement level. The current observations showed that the biogas residue might be considered as a protein feed source for pigs.

Introduction

There is a serious shortage of feed resources in China, and protein feed is particularly scarce (Zhao et al., 2009). In China, more than 60% of fishmeal used each year is imported, and for soybean meal this proportion reaches more than 70% (Zhang et al., 2013; Pan, 2013). Thus, the development of new protein feed sources has been an important concern for the sustained development of the feed and livestock industries. Based on local resource availability, Anhui Ruifuxiang Food Co., Ltd. (ARFCL) prepares alcohol from wheat using proprietary processes. Biogas residue is produced from liquid vinasse through centrifugation, flocculation, filtration, and biogas fermentation, after which protein content reaches 35% (Kang et al., 2014). With recent increases in the wheat processing capacity of ARFCL, its annual cumulative yield of biogas residue and scum has reached 40,000 t. If the slurry is left unused, significant investment must be made for innocuous treatment; therefore, the development of methods to process the biogas residue into nutrient-rich feed is a high-priority research focus.

The main protein constituent of biogas residue is gluten, which contains high levels of amino acids, with the exception of lysine. Glutamic acid is beneficial to animal digestive health and accounts for 30% of the total gluten content. Gluten has been shown to promote the growth of weaned piglets; in particular, it increased their average daily weight gain and improved the feed-to-gain ratio (Richert et al., 1994). Moreover, in comparison with plasma proteins and glutenine, gluten enhances the immune function of piglets. Because the safety and nutritive value of biogas residue as a source of animal feed have not been reported, we systematically evaluated these characteristics in animal models to provide an empirical basis for the reutilization of biogas residue as a feed source after innocuous treatment.

Materials and methods

Biogas residue was provided by Anhui Ruifuxiang Food Co., Ltd. and contained the following components: dry matter (DM, 91.39%), crude protein (CP, 35.79%), ether extract (EE, 8.60%), crude fiber (CF, 4.57%), crude ash (CA, 4.08%), nitrogen-free extract (NFE, 38.35%), calcium (Ca, 0.38%), and phosphorus (P, 0.6%).

All experiments were approved by the Institutional Animal Care and Use committee of Anhui Agricultural University. Normal Kunming mice (N = 176, 18 to 20 g) were purchased from the Laboratory Animal Center of Anhui Medical University (136 females and 40 males). The temperature of the mouse cage was maintained at 22 ± 3 °C with relative humidity of 55% and a 12:12 light:dark cycle. The mice were allowed free access to water. Before the test, the mice were allowed to adapt to the testing conditions for at least 7 days.

Experiment 1

The diet used in the acute toxicity testing was provided by the Laboratory Animal Center of Anhui Medical University. Ninety-six female mice were divided into 4 groups (1 control group and 3 treatment groups). There were 4 replicates of each group and 6 individuals in each replicate. Air-dried biogas residue was mixed with water at a ratio of 1:3 (w/v) and soaked at 4 °C for 24 h, after which it was filtered using 2-layer gauze and centrifuged for 10 min (5000 rpm). The biogas residue solutions were prepared at 1 g/mL, 5 g/mL, and 15 g/mL and administered intragastrically. The mice were fasted for 4 h before slurry administration, but were allowed free access to water. Prior to slurry administration, the mice were weighed and the average weight of each repli-
cate group was calculated. Group 1, Group 2, and Group 3 received 0.5 mL of the slurry solution at 1 g/mL, 5 g/mL, and 15 g/mL, respectively. The control group received 0.5 mL of distilled water. After the intragastric administration, the mice were fasted for 1 h, after which free intake of standard feed was allowed, and the behaviour of the mice was observed carefully. After 24 h, the mice were fasted for 12 hours with free access to water. After the fast, two mice were selected from each replicate of each group, an eye was removed from each mouse for blood sampling, and the mice were dissected. The remaining mice from each group were observed for a further 14 days to allow for the manifestation of delayed toxicity.

Experiment 2

For sub-acute and sub-chronic toxicity testing methods, see GB/T 23179-2008 and GB/T 21763-2008. First, 80 mice were divided into a control group and a biogas residue group for each test. There were 4 replicate groups of 10 subjects for each treatment (5 males and 5 females in each group). The formulated diets are shown in Table 1. On the 30th day of the sub-acute toxicity test, the mice were fasted (with free access to water) for 12 h before blood sampling. Two mice were selected from each replicate of each treatment group for blood sampling. Two mice were selected from each replicate and observed for a further 14 days, during which they received the control diet. In the sub-chronic toxicity test, sampling was performed on the 60th and 90th day using the method described above.

Experiment 3

For the digestion and metabolism test, 4 healthy Duroc × Landrace × Yorkshire (D×L×Y) cross-bred pigs weighing about 35 kg were used to study the nutritional value of the biogas residue. The metabolism test was divided into 2 periods, in which the test diet and only protein source was either a low-casein corn starch-based diet or the protein feed mixture. The composition and nutrient contents of the diets are shown in Table 2. Pigs were reared separately in metabolic cages. There was a 7-day adaptation phase, during which the pigs received a well-balanced diet. A T-shaped fistula was created surgically at the terminal ileum as described by Nyachoti et al. (2002). Postoperative management was conducted according to methods reported by Stein et al. (2006). During the test, daily feed allowance was set at 90% of maximum intake, which was divided into 2 equal meals at 08:00 and 17:00 h. Sufficient clean water was available at all times. The test was divided into 2 periods of 9 days each, which included a 4-d preliminary feeding phase, a 3-d fecaluria collecting phase, and a 2-d phase during which digesta was collected from the terminal ileum. The digesta collection was performed according to the method reported by Stein et al. (1999). For the 1st and 2nd test period, four pigs received biogas residue or a low-casein corn starch-based diet, respectively. Samples of faeces, urine, and digesta were sealed and stored at -20°C (Li et al., 2013). At the end of the feeding test, faecal samples were unfrozen and air-dried. Digesta samples were unfrozen and freeze-dried with a vacuum freeze dryer.

The digesta samples were assayed after they were dampened with water for 24 h. For urine testing, 5 mL of urine was dropped onto a piece of filter paper, which was dried at 65 °C for 24 h. After dampening the filter paper with water, it was weighed and the urine energy was calculated using the automatic Isoperibol Calorimeter (Model 6300, Parr Instrument Company, Moline, IL, USA). Amino acid samples were treated by acid hydrolysis (6N HCl at 110 °C for 24 h), oxidation hydrolysis, or alkali hydrolysis (AOAC, 1998). Amino acid content (with the exception of tryptophan) was detected using an automatic amino acid analyser.

Table 1. Composition and nutrient levels of mouse diets (air-dry basis).

| Ingredients, % | Control group | Biogas residue group |
|----------------|---------------|---------------------|
| Corn starch    | 65.80         | 65.80               |
| Soybean meal   | 20.00         | 20.00               |
| Biogas residue | 10.00         | 10.00               |
| Fishmeal       | 1.80          | 1.80                |
| Limestone powder| 0.40         | 0.40                |
| NaCl           | 1.00          | 1.00                |
| Soybean oil    | 1.00          | 1.00                |
| Compound premix°| 0.90        | 1.00                |
| Total          | 100.00        | 100.00              |

Nutrient levels, %

| Nutrient, % | Control group | Biogas residue group |
|-------------|---------------|---------------------|
| Crude protein | 19.97      | 19.87               |
| Ether extract | 4.96        | 6.20                |
| Crude fibre  | 2.27         | 3.25                |

*Compound premix provided the following per kilogram of feed: vitamin A, 3000 U; vitamin D₃, 3000 U; vitamin E, 12 mg; vitamin K₃, 1.2 mg; vitamin B₁, 1 mg; vitamin B₂, 4 mg; vitamin B₆, 2 mg; vitamin B₁₂, 0.02 mg; nicotinic acid, 20 mg; folate, 1 mg; pantothenic acid, 9.2 mg; choline, 0.4 g; Se, 0.33 g; Cu, 0.1 g; Fe, 0.25 g; Zn, 0.12 g; Mn, 0.045 g; I, 0.7 mg.

Table 2. Composition and nutrient levels of pig diets (air-dry basis).

| Ingredients, % | Biogas residue group | Casein group |
|----------------|----------------------|--------------|
| Corn starch    | 35.89                | 63.24        |
| Sucrose        | 18.00                | 18.00        |
| Soybean oil    | 3.00                 | 3.00         |
| Biogas residue | 38.59                | 38.59        |
| Cellulose      | 5.00                 | 5.00         |
| Casein, CaHPO₄ | 0.36                 | 0.36         |
| Cr₂O₃          | 0.25                 | 0.25         |
| Compound premix°| 4.00            | 4.00         |
| Nutrient levels|                     |              |
| Gross energy, kcal/kg | 3648.25       | 3633.48     |
| Crude protein, % | 15.01                | 4.22         |
| Ca, %          | 0.68                 | 0.94         |
| P, %           | 0.45                 | 0.45         |

*Compound premix provided the following per kilogram of feed: vitamin A, 4000 U; vitamin D₃, 2000 U; vitamin E, 11.2 mg; vitamin K₃, 1.2 mg; vitamin B₁, 2.8 mg; vitamin B₂, 1.6 mg; nicotinic acid, 14 mg; pantothenic acid, 8 mg; choline, 0.32 mg; Se, 0.28 mg; Cu, 0.1 g; Fe, 0.12 g; Zn, 0.12 g; Mn, 0.032 g; I, 0.28 g.
and sub-chronic toxicity tests. Initial body observation periods of the acute, sub-acute, and dead mice were dissected immediately. The indicators included in a method similar to that of Williams et al. (1982).

Indicators and calculating methods

Toxicity test

The appearance and behaviours of the mice were observed every day. Each group of mice with toxicosis symptoms was reared separately, and dead mice were dissected immediately. The mice were weighed weekly during the observation periods of the acute, sub-acute, and sub-chronic toxicity tests. Initial body weight and the body weight on the 30th day and 90th day of observation were also recorded. The organ coefficients of the mice were calculated for this purpose, as well as in those found dead of other causes.

Serum biochemical indicators were assayed in the acute toxicity test after 24 hours, on the 14th and 30th days of the sub-acute toxicity test, and on the 60th and 90th days of the sub-chronic toxicity test. The indicators included asparatate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein (TP), albumin (Alb), glucose (GLU), creatinine (Cr), and total cholesterol (TCH). The apparent digestibility of amino acids in the terminal ileum was calculated using the following formula:

\[
d_{a} = 100 - \frac{(Ia 	imes Af)}{(Id 	imes Ad)} \times 100
\]

where \(d_a\) was the apparent digestibility (%), \(I_a\) was the amino acid content of the diet (%), \(I_f\) was the amino acid content of the digesta; and \(A_d\) was the amino acid content in the test diet (%) (Sauer et al., 1991).

The true amino acid digestibility in the terminal ileum was calculated using the following formula:

\[
d_{t} = d_{a} + D\times I_{DA}
\]

where \(d_t\) was the true amino acid digestibility (%), \(d_a\) was the apparent digestibility (%), \(D\) was the amount of excreted endogenous amino acids (%), and \(I_{DA}\) was the amino acid content of the dry matter of the test diet (kg).

The amount of excreted endogenous amino acids was calculated using the following formula:

\[
A_{e} = \frac{100 - D_{da}}{100} \times I_{DA}
\]

where \(A_e\) was the amount of excreted endogenous amino acids in the diet, which was calculated using the following equation:

\[
D_{da} = \frac{ME_{diet} - (ME_{sugar} \times \%sugar) - (ME_{starch} \times \%starch) - (ME_{soybean} \times \%protein)}{ME_{soybean} \times \%protein}
\]

where \(ME_{sugar}\) was the percentage of sugar, \(ME_{starch}\) was the percentage of starch, \(ME_{soybean}\) was the percentage of soya bean oil, \(ME_{soybean}\) was the ME of soybean oil, \(\%protein\) was the percentage of protein in the diet, and \(D_{da}\) was the digestible energy of the diet, which was calculated using the following equation:

\[
D_{da} = \frac{GE - FE}{UE}
\]

where \(GE\) was the gross energy of the diet and \(FE\) was the faecal energy.

The metabolic energy (ME) of the biogas residue was calculated using the following equation:

\[
ME_{diet} = GE - FE
\]

Table 3. Serum biochemical indexes (24 h) and average weekly weight gain after treatment with biogas residue concentrate solution.

| Indicator       | 0 g/mL     | 1 g/mL     | 5 g/mL     | 15 g/mL    |
|-----------------|------------|------------|------------|------------|
| AST, U/L        | 168.20±19.29 | 154.00±18.91 | 198.17±57.62 | 149.00±23.11 |
| ALT, U/L        | 52.20±14.55  | 39.50±9.85  | 45.83±6.06  | 38.83±4.71  |
| ALP, U/L        | 195.20±63.20 | 152.33±74.74 | 218.67±57.52 | 204.50±37.12 |
| BUN, mmol/L     | 9.82±1.27   | 9.40±1.25   | 9.94±1.57   | 9.46±1.04   |
| TP, g/L         | 59.14±4.84  | 58.50±1.62  | 57.13±5.72  | 57.83±2.17  |
| ALB, g/L        | 36.12±1.88  | 34.30±3.15  | 35.73±2.52  | 35.22±2.17  |
| GLU, mmol/L     | 2.06±0.57   | 1.96±0.77   | 2.49±0.47   | 2.00±1.00   |
| Cr, μmol/L      | 10.00±1.87  | 10.33±1.21  | 10.17±1.47  | 9.50±2.17   |
| TCH, mmol/L     | 2.64±0.35   | 2.01±0.41   | 2.61±0.32   | 2.46±0.32   |
| AWG             | 2.08±1.91   | 3.65±1.42   | 2.08±1.95   | 3.75±1.02   |
| First week, g/mouse | 0.42±1.44  | 1.25±2.50   | 0.42±1.95   | 1.25±1.77   |

AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; TP, total protein; ALB, albumin; GLU, glucose; Cr, creatinine; TCH, total cholesterol, AWG, average weekly weight gain.
Statistical analysis

SAS 9.0 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis (except Exp. 3). Group differences were evaluated using one-way analysis of variance (ANOVA) or Student's t-test with a significance threshold of P < 0.05. One-way ANOVA with repeated-measures was used for Exp. 2. The results are expressed as mean ± SD. For all tests, a cage served as the experimental unit.

Results

Acute toxicity test

After the administration of the biogas residue to mice, behaviour and faecal colour were unchanged for 3 hours, and no subjects died. One mouse treated with 15 g/mL biogas residue died within the first 24 hours after administration. No obvious pathological changes in the heart, liver, spleen, lung, or kidney were observed during the dissection. Two mice treated with 1 g/mL biogas residue and 1 mouse treated with 5 g/mL biogas residue died during the 14-day observation period. All groups had mortality rates of less than 50%; therefore, because the administered dose of the biogas residue was greater than 20,000 mg/kg, the median lethal dose (LD₅₀) of the biogas residue was determined to be greater than 20,000 mg/kg. No significant differences were found among the groups in the analyses of serum biochemical indicators 24 hours after biogas residue administration and average weekly weight gain during the first 2 weeks of observation (P>0.05) (Table 3).

Sub-acute and sub-chronic toxicity test

During the sub-acute and sub-chronic toxicity tests, the mice in the treatment and control groups did not show abnormal behaviour. Two mice from the control group and 3 mice from the biogas residue group died within the first 30 days of the sub-acute toxicity test. Mild blood congestion was found in the lungs of 1 mouse from each group during dissection. During the 2-week observation period, all mice acted normally and no mice died. No significant differences were found between the groups in the analysis of serum biochemical indicators on the 30th day and the 2-week observation period after the 30th day of the sub-acute toxicity test, and the weights of the groups on the 30th day were similar (P>0.05) (Tables 4 and 5). The spleen index of the biogas residue group was significantly greater (P<0.05) than that of the control group in the sub-acute toxicity test, but splenomegaly did not occur. Normal visceral colour was observed in the biogas residue group and in the control group. On the 60th day of the sub-chronic toxicity test, the serum urea nitrogen concentration of the biogas residue group was significantly less than that of the control group (P<0.05) and the remaining serum biochemical indicators did not show significant differences (P>0.05). On the 90th day of the test, the serum AST and ALT concentrations of mice in the biogas residue group were considerably greater than those of the control group (P<0.05), while other biochemical indicators showed no significant differences (P>0.05). On the 60th and 90th day of the test, visceral index and body weight were similar in the biogas residue and control groups (P>0.05).

Time significantly impacted the levels of AST, ALP, TP, ALB, and GLU, as well as the heart index, spleen index, and kidney index (Table 4). During the period from the 30th day of the test to the 90th day of the test, the level of AST first increased and then decreased in the biogas residue group, while the level of AST gradually decreased in the control group. In addition, the biogas residue and control groups showed a significant difference in AST level on the 90th day of the test. During the period from the 30th day of the test to the 90th day of the test, the control and biogas residue groups showed similar changes: the ALP level gradually decreased, the GLU level gradually increased, the TP level increased first and then decreased, and the ALB level, heart index, spleen index, and kidney index decreased first and then increased. With the exception of the spleen index, the indexes mentioned above were correlated more strongly with time than with diet. However, analysis of the effects of time and diet on the spleen index showed a significant interaction (P<0.05). In addition, BUN level was significantly affected by changes in diet rather than time, and the diet-induced changes in BUN level were not affected by time. During the period from the 30th day of the test to the 90th day of the test, the BUN level of the control group gradually decreased, while the BUN level of the biogas residue group first decreased and then increased. In addition, the biogas residue and control groups showed a significant difference in BUN level on the 60th day of the test. Although the biogas residue and control groups showed a significant difference in ALT level on the 90th day of the test, statistical analysis indicated that this difference was not significantly associated with time or diet.

Digestion and metabolism tests

The digestible energy and metabolic energy of the biogas residue, apparent digestibility of its dry matter, and apparent and true digestibility of its crude protein are shown in Table 6. The digestibility of the amino acids in the biogas residue in pigs is shown in Table 7. The apparent digestibility of arginine (77.51%) was greater than that of tryptophan (72.93%) and lysine (72.29%), which were more digestible than methionine (55.25%).

Discussion

Toxicity tests

ALT and AST are involved in transamination reactions and reflect the status of protein synthesis and catabolic metabolism. Serum ALT and AST are indicators of the health status of the liver. When cells and mitochondria are damaged, ALT and AST are released into the blood, resulting in a measurable increase in their abundance and activity (Bogin et al., 1997; Zhu et al., 1999). In the acute toxicity test, the mice did not show abnormal behaviour, and the deaths in each group did not occur in a dose-related manner. In addition, dissections showed that the mice that died naturally had normal organ texture and colour. There were no significant differences in serum biochemical indicators 24 hours after treatment, and the average weight gain during the 14-day observation period was similar across the groups. These acute toxicity testing results showed that the biogas residue was harmless to the mice. The mortality rates at the selected doses indicate that the LD₅₀ of the biogas residue is greater than 20,000 mg·kg⁻¹, which meets the criteria for non-toxicity published by the World Health Organization (WHO) and the Ministry of Health of the People’s Republic of China (MoH) (2003).

Organ coefficient is the ratio of the wet weight of an organ to the weight of the animal, and it is a sensitive, effective, and low-cost indicator of the degree of organ injury produced by a test substance. Peripheral immune organs such as the spleen and lymph nodes are involved in antigen recognition and lymphocyte activation (Kindt et al., 2006), but the spleen is the primary immunity organ after maturation of the organism. The spleen contains T lymphocytes, B lymphocytes, and macrophages, which are involved in humoral and cellular immunity (Dvorak and Sciuto, 2004). As shown in Table 4, time and the interaction between time and diet significantly affected the spleen index, which may indicate
Table 4. Serum biochemical and organ indexes from mice in the sub-acute toxicity and sub-chronic toxicity tests.

|                      | Control       | Biogas residue | Pooled SEM | D   | T   | D×T  |
|----------------------|---------------|----------------|------------|-----|-----|------|
| AST, U/L             |               |                |            |     |     |      |
| Sub-acute d 30       | 167.38        | 180.88         | 11.83      | 0.100 | 0.045 | 0.131 |
| Sub-acute observation period | 128.75 | 135.25         | 10.12      |     |     |      |
| Sub-chronic d 60     | 139.38        | 131.88         | 7.24       |     |     |      |
| Sub-chronic d 90     | 112.13\abc    | 151.63\abc     | 9.02       |     |     |      |
| ALT, U/L             |               |                |            |     |     |      |
| Sub-acute d 30       | 67.50\abc     | 52.50          | 5.65       | 0.957 | 0.146 | 0.072 |
| Sub-acute observation period | 42.50 | 41.88          | 4.72       |     |     |      |
| Sub-chronic d 60     | 44.38\abc     | 44.00          | 4.35       |     |     |      |
| Sub-chronic d 90     | 37.25\abc     | 53.38\abc      | 4.03       |     |     |      |
| ALP, U/L             |               |                |            |     |     |      |
| Sub-acute d 30       | 152.88        | 202.00         | 19.20      | 0.263 | 0.004 | 0.411 |
| Sub-acute observation period | 152.88 | 202.00         | 9.03       |     |     |      |
| Sub-chronic d 60     | 134.00        | 148.75         | 11.79      |     |     |      |
| Sub-chronic d 90     | 106.50\abc    | 105.63\abc     | 10.44      |     |     |      |
| BUN, mmol/L          |               |                |            |     |     |      |
| Sub-acute d 30       | 9.14          | 8.52           | 0.40       | 0.024 | 0.239 | 0.376 |
| Sub-acute observation period | 9.38 | 10.00          | 0.56       |     |     |      |
| Sub-chronic d 60     | 8.95\abc     | 6.82\abc       | 0.45       |     |     |      |
| Sub-chronic d 90     | 8.85          | 8.15           | 0.33       |     |     |      |
| TP, g/L              |               |                |            |     |     |      |
| Sub-acute d 30       | 69.71\abc    | 68.24\abc      | 1.67       | 0.626 | 0.008 | 0.929 |
| Sub-acute observation period | 65.86 | 75.96\abc     | 1.63       |     |     |      |
| Sub-chronic d 60     | 70.07\abc    | 70.92\abc      | 0.92       |     |     |      |
| Sub-chronic d 90     | 36.38         | 33.52          | 1.01       | 0.114 | 0.036 | 0.552 |
| ALB, g/L             |               |                |            |     |     |      |
| Sub-acute d 30       | 35.13         | 37.49          | 0.70       |     |     |      |
| Sub-acute observation period | 39.64 | 38.05          | 0.74       |     |     |      |
| Sub-chronic d 60     | 37.49         | 36.93          | 0.69       |     |     |      |
| GLU, mmol/L          |               |                |            |     |     |      |
| Sub-acute d 30       | 1.66\abc     | 1.13\abc       | 0.28       | 0.119 | 0.000 | 0.988 |
| Sub-acute observation period | 6.02 | 5.29          | 0.37       |     |     |      |
| Sub-chronic d 60     | 2.65\abc     | 2.27\abc       | 0.32       |     |     |      |
| Sub-chronic d 90     | 5.53\abc     | 5.04\abc       | 0.28       |     |     |      |
| Cr, mol/L            |               |                |            |     |     |      |
| Sub-acute d 30       | 13.50         | 11.50          | 0.65       | 0.431 | 0.200 | 0.256 |
| Sub-acute observation period | 8.63 | 8.75          | 0.39       |     |     |      |
| Sub-chronic d 60     | 13.63         | 14.75          | 0.86       |     |     |      |
| Sub-chronic d 90     | 14.00         | 12.75          | 0.64       |     |     |      |
| TCH, mmol/L          |               |                |            |     |     |      |
| Sub-acute d 30       | 2.58          | 2.47           | 0.18       | 0.591 | 0.130 | 0.970 |
| Sub-acute observation period | 2.00 | 2.43          | 0.13       |     |     |      |
| Sub-chronic d 60     | 2.48          | 2.34           | 0.13       |     |     |      |
| Sub-chronic d 90     | 2.12          | 2.07           | 0.16       |     |     |      |
| Heart index, %       |               |                |            |     |     |      |
| Sub-acute d 30       | 0.63\abc     | 0.58\abc       | 0.03       | 0.953 | 0.000 | 0.307 |
| Sub-chronic d 60     | 0.43\abc     | 0.47\abc       | 0.02       |     |     |      |
| Sub-chronic d 90     | 0.43\abc     | 0.44\abc       | 0.01       |     |     |      |
| Liver index, %       |               |                |            |     |     |      |
| Sub-acute d 30       | 3.93\abc    | 4.17\abc       | 0.12       | 0.094 | 0.000 | 0.876 |
| Sub-chronic d 60     | 3.51\abc    | 3.65\abc       | 0.07       |     |     |      |
| Sub-chronic d 90     | 4.35\abc    | 4.58\abc       | 0.09       |     |     |      |
| Spleen index, %      |               |                |            |     |     |      |
| Sub-acute d 30       | 0.40\abc    | 0.52\abc       | 0.03       | 0.999 | 0.002 | 0.040 |
| Sub-chronic d 60     | 0.35\abc    | 0.27\abc       | 0.02       |     |     |      |
| Sub-chronic d 90     | 0.35\abc    | 0.32\abc       | 0.02       |     |     |      |
| Lung index, %        |               |                |            |     |     |      |
| Sub-acute d 30       | 0.81          | 1.09           | 0.11       | 0.078 | 0.101 | 0.887 |
| Sub-chronic d 60     | 0.54          | 0.69           | 0.10       |     |     |      |
| Sub-chronic d 90     | 0.60          | 0.75           | 0.08       |     |     |      |
| Kidney index, %      |               |                |            |     |     |      |
| Sub-acute d 30       | 1.16\abc   | 1.24           | 0.04       | 0.200 | 0.000 | 0.990 |
| Sub-chronic d 60     | 0.96\abc   | 1.03           | 0.04       |     |     |      |
| Sub-chronic d 90     | 1.07          | 1.15           | 0.04       |     |     |      |

*Means within the same row and column with different superscript letters differ significantly (P<0.05). D, diet; T, time; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; TP, total protein; ALB, albumin; GLU, glucose; Cr, creatinine; TCH, total cholesterol.
an influence of these factors on the immune function of the body. The spleen index of the mice treated with the biogas residue was increased significantly in the sub-acute toxicity test, but the spleen was not enlarged. Furthermore, pathological changes were not found during visual assessments of the organs. These results suggest that non-specific immune function may be enhanced by biogas residue administration.

In the sub-acute and sub-chronic toxicity tests, all mice behaved normally. The measurements of serum biochemical indicators in the control group and treatment group did not show significant differences on the 30th day of the sub-acute test or at the end of the observation period. Blood congestion in the lung was found in the treatment and control groups, indicating that the mortality in the treatment groups did not occur as a result of slurry exposure. The results from our assessments of the heart index, liver index, TP level, and GLU level in the biogas residue and control groups on the 30th and 90th day of the test indicate that the biogas residue did not damage the heart or liver. The high ALP level on the 30th day of the test in the biogas residue group and the control group may have been due to the expected increase in the number of osteoblasts in the mouse during periods of rapid growth (Zhou et al., 2014). As the testing period elapsed, the ALP level gradually decreased to its normal physiological level (Hu et al., 2007). Creatinine and urea nitrogen concentrations are commonly used indicators of renal function that reflect the health status of the renal parenchyma and the degree of proteolysis (Kiliç and Akay, 2008). Creatinine and urea nitrogen are metabolites of creatine and protein, respectively, and they are filtered by the glomerulus and excreted in the urine. When glomerular filtration is impaired, serum creatinine and urea nitrogen levels increase. As shown in Table 4, the changes in urea nitrogen content were caused by diet rather than time. On the 60th day of the sub-chronic toxicity test, the serum urea nitrogen level in biogas residue group was decreased, but was still in the normal range, while the creatinine content and organ coefficients showed no obvious changes. This result shows that the biogas residue is harmless to the kidney. On the 90th day of the sub-chronic toxicity test, ALT and AST levels were increased significantly, but they remained in the normal range (Hu et al., 2007). Dissections showed that the texture and colour of each organ were normal and that necrosis did not occur on the organ surface. Furthermore, the organ coefficients were similar in the treatment and control groups. These results verify the harmlessness of biogas residue for the mice.

The repeated-measures analysis showed that there were no significant effects of diet on most of the indexes assessed in this study; the significant changes in the indexes were mainly influenced by time. The significant influence of time on most of the indexes changed in this study may have been due to the different ages of the mice used in the experiments (Hu et al., 2007; Lu et al., 2007). Although some biochemical indicators in the mice treated with the biogas residue were changed, they remained within their normal ranges. Therefore, the biogas residue did not adversely affect the growth of exposed mice, and biogas residue is a safe additive (at a 20% replacement level) in animal feed.

### Table 5. Average mouse weight in the sub-acute toxicity and sub-chronic toxicity tests.

|                | Control            | Biogas residue |
|----------------|--------------------|----------------|
| Initial weight, g | 18.25±0.87         | 18.13±1.31     |
| Weight at day 30, g | 29.25±2.50         | 27.35±3.26     |
| Final weight, g    | 35.11±3.18         | 34.92±5.69     |

### Table 6. Digestibility and availability of biogas residue in pigs.

|                  | Biogas residue |
|------------------|----------------|
| Digestible energy, MJ/kg DM | 13.84±0.59    |
| Metabolic energy, MJ/kg DM    | 13.61±0.83    |
| DM apparent digestibility, %DM | 86.00±1.69    |
| CP apparent digestibility, %DM | 77.33±3.14    |
| CP true digestibility, %DM    | 82.98±3.09    |

DM, dry matter; CP, crude protein.

### Table 7. Amino acid content and digestibility of biogas residue (dry matter basis).

| Amino acid     | Amino acid content, % | Apparent digestibility, % | True digestibility, % |
|----------------|-----------------------|---------------------------|-----------------------|
| Arg            | 1.62                  | 77.51±1.46                | 77.57±1.48            |
| His            | 0.76                  | 67.22±2.57                | 67.30±2.59            |
| Ile            | 1.30                  | 64.92±2.82                | 65.01±2.83            |
| Leu            | 2.46                  | 69.27±1.95                | 69.34±1.96            |
| Lys            | 1.70                  | 72.29±2.08                | 72.37±2.10            |
| Met            | 0.68                  | 55.25±2.01                | 55.32±1.98            |
| Trp            | 0.33                  | 72.93±5.41                | 73.76±5.40            |
| Phe            | 1.55                  | 68.23±2.01                | 68.31±2.02            |
| Thr            | 1.34                  | 60.40±3.26                | 60.50±3.28            |
| Val            | 1.71                  | 65.98±1.92                | 66.06±1.93            |
| Ala            | 2.04                  | 57.26±4.04                | 57.34±4.04            |
| Asp            | 2.84                  | 62.93±2.53                | 63.02±2.55            |
| Cys            | 0.68                  | 62.49±3.81                | 62.60±3.80            |
| Glu            | 4.97                  | 68.00±0.99                | 68.08±1.00            |
| Gly            | 1.62                  | 55.80±5.33                | 55.88±5.33            |
| Ser            | 1.50                  | 63.59±2.83                | 63.72±2.84            |
| Tyr            | 1.12                  | 67.41±1.15                | 67.50±1.15            |

Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Trp, tryptophan; Phe, phenylalanine; Thr, threonine; Val, valine; Ala, alanine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gly, glycine; Ser, serine; Tyr, tyrosine.
gas residue in pigs was 13.84 MJ/kg, while the metabolic energy was 13.61 MJ/kg, which were each greater than the corresponding values for fishmeal. The digestible energy of the biogas residue is 2.40% lower than that of wheat, and it is 2.95% lower than that of soybean meal.

The composition and nutrient digestibility of feed are important determining factors of its nutritive value. Digestibility is influenced by the intrinsic properties of feed, processing technology, and the digesting animals (Zhao et al., 2009). Protein digestibility measurements indicated that the apparent digestibility of crude protein in the biogas residue was 77.33% and the true digestibility was 82.98%, which were each less than the corresponding values for soybean meal and fishmeal. The crude protein in the biogas residue is low-digestibility protein, which may be a result of the use of polycrylicamide as a flocculant during processing of the liquid vinasse. The nitrogen contained in the crude protein cannot be utilized effectively by the ingesting organism.

The digestibility of amino acids is usually used to assess the nutritive value of proteins in feed for monogastric animals. Compared with apparent digestibility, the true digestibility of amino acids in the terminal ileum better reflects the nutritive value of ingredients in the feed. The standard digestibility values for soybean meal amino acids in the ileum given in the Chinese Feed Database (2012) was used as reference data. The apparent and true digestibility of biogas residue amino acids ranged from 55 to 77%, which was less than the standard ideal digestibility of soybean meal. The true digestibility of lysine and methionine were 17.78% and 37.84% less than the reference standards, respectively. Our results show that the energy digestibility of the biogas residue is similar to that of soybean meal, while the digestibility of biogas residue crude protein, as well as that of amino acids, is lower than that of soybean meal. Because the biogas residue is a waste product, economic benefits can be achieved by using it to partially replace soybean meal or other types of protein feed. Furthermore, the novel approach for the reutilization of organic wastes described herein could lead to significant environmental benefits by removing a substantial source of waste.

Conclusions

The biogas residue produced as a waste product in the manufacture of biogas from wheat is safe and had no adverse effects on the growth of mice when ingested. The current observations demonstrate that biogas residue from wheat manufacturing may be used as a protein feed source for pigs.

References

Adeola, O., 2001. Digestion and balance techniques in pigs. Swine nutrition. 2nd ed. CRC Press, New York, NY, USA.

AOAC, 1998. Official methods of analysis. 16th ed. Association of Analytical Chemists, Arlington, VA, USA.

Bogin, E., Peh, H.C., Avidar, Y., Israel, B., Kevkhsyay, E., Lombardi, P., Cahaner, A., 1997. Sex and genotype dependence on the effects of long-term high environmental temperatures on cellular enzyme activities from chicken organs. Avian Pathol. 26:511-524.

Chinese Feed Database, 2012. Tables of feed composition and nutritive values in China. China Feed 21:34-43.

Dvorak, A.M., Sciuto T.E., 2004. Mouse spleen basophils. Int. Arch. Allergy. Immun. 134:332-333.

Hu, J.H., Lu, S.M., Che, L.P., 2007. A probe into the weight and utilization of lysine fermented protein meal in growing-finishing pigs. J. Anim. Sci. 84:853-860.

Kang, L.H., Li, L.L., Si, X.Y., Li, B., Guo, W.J., Mu, H., Ding, X.L., Xu, F.Z., 2014. Screening of the strains and antioxidant activity of small peptide from solid-state fermentation of the residue from wheat alcohol processing. Food Ferment. Ind. 40:72-76.

Kiliç A., Akay, M.T., 2008. A three generation study with genetically modified Bt corn in rats: biochemical and histopathological investigation. Food Chem. Toxicol. 46:1164-1170.

Kindt, T.J., Osborne, B.A., Goldsby, R.A., 2006. Kuby immunology. 6th ed. W.H. Freeman and Co Ltd., Coventry, UK.

Li, S.F., Niu, Y.B., Liu, J.S., Lu, L., Zhang, L.Y., Ran, C.Y., Feng, M.S., Du, B., Deng, J.L., Luo, X.G., 2013. Energy, amino acid, and phosphorus digestibility of phytase transgenic corn for growing pigs. J. Anim. Sci. 91:298-308.

Lu, S.M., Hu, J.H., Che, L.P., 2007. The weight of main organs coefficient in 10 kinds of common SPF rats and mice. Lab. Anim. Sci. 24:12-16.

National Standard of the People’s Republic of China, 2003. Procedures for toxicological assessment of food. Ministry of Health of the People’s Republic of China ed., Beijing, China.

Nyachoti, C.M., McNeilage-Van de Wiele, E.M., De Lange, C.F.M., Gabert, V.M., 2002. Evaluation of the homoarginine technique for measuring true ileal amino acid digestibilities in pigs fed a barley-canola meal-based diet. J. Anim. Sci. 80:440-448.

Pan, R., 2013. A review of soybean meal market in the years of 2012 and the expectation for 2013. Feed China 2013:18-19.

Richert, B.T., Hancock, J.D., Morrill, J.L., 1994. Effects of replacing milk and soybean products with wheat gluten on digestibility of nutrients and growth performance in nursery pigs. J. Anim. Sci. 72:151-159.

Sauer, W.C., Mosenthin, R., Ahrens, F., Den Hartog, L.A., 1991. The effect of source of fiber on ileal and faecal amino acid digestibility and bacterial nitrogen excretion in growing pigs. J. Anim. Sci. 69:4070-4077.

Stein, H.H., Aref, S., Easter, R.A., 1999. Comparative protein and amino acid digestibilities in growing pigs and sows. J. Anim. Sci. 77:1169-1179.

Stein, H.H., Gibson, M.L., Pedersen, C., Boersma, M.G., 2006. Amino acid and energy digestibility in ten samples of distillers dried grain with solubles fed to growing pigs. J. Anim. Sci. 84:853-860.

Williams, C.H., David, D.J., Lissner, O., 1962. The determination of chromic oxide in faecal samples by atomic absorption spectrophotometry. J. Agr. Sci. 59:381-385.

Zhang, L.G., Du, F.J., Ji, X.F., 2013. The main feed raw material rising concerns about external dependency. Feed China 2013:19-21.

Zhou, Y., Chen, D.W., Yu, B., Zeng, Q.F., Wu, X.Q., 2009. Evaluation of nutritional value and utilization of lysine fermented protein meal in growing-finishing pigs. J. Anim. Nutr. 21:363-370.

Zhou, Y., Gu, L.Y., Hu, H.J., Zhan, X.A., Pu, Q.H., 2014. Effects of feeding amount of yellow rice wine lees (YRWL) on the growth performance, nutrient digestibility and serum biochemical index of finishing pigs. Feed China 2014:17-20.

Zhu, M., Lin, K.F., Yeung, R.Y., Li, R.C., 1999. Evaluation of the protective effects of Schisandra chinensis on phase I drug metabolism using a CCl4 intoxication. J. Ethnopharmacol. 67:61-68.

Ital J Anim Sci vol.14:2015