Contents lists available at Science-Gate

International Journal of Advanced and Applied Sciences

Journal homepage: http://www.science-gate.com/IJAAS.html

# A predigestion process applied to wheat straw to increase its water-soluble mass

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ARTICLE INFO

*Article history:*
Received 25 February 2020
Received in revised form
12 June 2020
Accepted 14 June 2020

*Keywords:*
Wheat straw
Microflora
Predigestion
Water-solubles

ABSTRACT

Wheat straw predigestion is needed for the industrial bioprocess and ruminant feeding. We aimed to search for some predigestion conditions for wheat straw. 500ml of water was used as a moistener for each 100g of the chopped straw sample. For each sample, 2g NaOH as alkalizer; 3g citric acid as neutralizer; a mix of 1g $KH_2PO_4$, 1g $(NH_4)SO_4$, 0.3g $CaCl_2$, 0.2g $MgSO_4$, 2g powdered molasses and 1g inactive dry yeast as supplements were used. Inoculation culture was non-selected total flora of rumen remained live after consecutive treatments of 30 min of alkali (NaOH, pH 10) and 30 min of acid (3% citric acid, pH 4) environments. The samples were incubated aerobically at 37$^0$C for 14 days, and the analyses were made at days 0, 7, and 14. Hot alkali and neutralizer determined to be the most effective on predigestion when used consecutively. The 15 to 20% increases were determined from the water-soluble mass of the samples incubated for 7 days. No net effect of the supplements and inoculant on the predigestion was determined. From the dominant aerobic microflora of the predigested samples, some opportunistic and/or sporadic pathogenic aerobic Gram+ ve bacteria were identified by using VITEK 2 GP identification system. As a result, it was determined that 2g NaOH in hot water for 7 days of microbial predigestion of 100g chopped straw appeared to be sufficient. Nevertheless, the hygiene aspect of such bacteria in the predigestion applications have to be intensively investigated in further studies. Acceptability, digestibility, and rumen health effects of such predigested straw have also to be tested when it was aimed to prepare feed materials.



## 1. Introduction

Wheat straw, as a carbohydrate-rich material, has a large potential as a source of dietary energy for ruminants (Gado et al., 2017). However, it is well known that wheat straw has a low feeding value due to its depleted protein and energy, as well as high fiber content, and therefore, results in poor animal intake (Hart et al., 2008). Some biotechnological approaches for industrial productions of biomaterials and biofuels, some suitable microbes, have been employed for the predigestion of straw (Shi et al., 2018). The limited accessibility of lignocellulose for a microbial attack is also problematic in biotechnologies used for the fermentation of straw (Novy et al., 2015). Many physical, chemical and biological pretreatment procedures have been used to increase the accessible surface area, to modify the crystalline structure or partially depolymerize cellulose, to solubilize hemicelluloses and/or lignin (Cassells et al., 2017).

The essential physical treatment method for plant material is particle size reduction. It results in an enlargement of the specific surface area and a release of intracellular components (Gallegos et al., 2017). One of the most promising chemical methods is the pre-treatment of lignocelluloses with acids and/or alkalis. These methods have some disadvantages since they are costly, low in effectiveness, not environmentally friendly, and also require the application of technology. These factors limit their application, particularly at small farm levels (Jiang et al., 2016).

Hot water or steam moistening is needed for the start of the microbial treatment of straw. Acids and alkalis are also combined in that water supply

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https://doi.org/10.21833/ijaas.2020.10.013
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(Sträuber et al., 2015). However, microorganisms performing anaerobic fermentation of such pretreated substrate are generally not adapted to strong acid or alkaline conditions (Jiménez et al., 2014; Zhang et al., 2017). This could be circumvented by neutralization of the substrate after acid or alkaline pretreatment. However, the neutralization step would need additional chemicals and is, therefore, cost-intensive. To improve the hydrolysis step of anaerobic fermentation of wheat straw, more than one pretreatment and/or fermentation procedures have been used in combinations (Mahesh and Mohini, 2013).

Many bacterial and fungal species grow on moist substrates under aerobic conditions by solid-state fermentation (SSF) and have the ability to degrade fiber contents (Kanyinji and Moonga, 2014). Although used as a substrate for microbial growth, wheat straw has inherently poor levels of these macro elements. For enrichment, the content of ensiled straw for biodegradation, urea, molasses, ammonia, nitrates, lime, and some other ingredients have been added (Oladosu et al., 2016).

It has been stated that culturable microbes in the rumen account for less than 1% of the total microbial species (McCabe et al., 2015). The composition of the bacteria found in the rumen is differentiated by a number of factors. For example, diet can alter the dominance of different bacterial strains (Popova et al., 2019).

To increase digestibility by ruminants, the current achievements in industrial microbiology have started to apply to make semi-digested feed material from roughages at the in-vitro environment of farms. Now, the researchers are more focused on the subject than ever. Nevertheless, the role of aerobes in the in-vitro forage digestion process has not been investigated in detail yet. To make an environmentally friendly, cost-effective, simple, and applicable at farm level predigestion process of wheat straw, we aimed to search for an aerobic fermentation techniqueusing a semi-selected ruminal microflora by combining some pre-treatment applications.

## 2. Materials and methods

### 2.1. Straw samples

A total of 20kg wheat straw samples in the bulk storage of feed of dairy cattle, which was_formerly chopped to 2-5cm length, was taken from a local dairy farm and used in the whole experiments.

### 2.2. Inoculation Culture

The culture was prepared from rumen microflora of newly slaughtered adult cattle during slaughter process in a local abattoir in Nicosia/Cyprus, the surface of rumens of five cows were disinfected by using sterile cotton sponges moistened with a 70% ethanol, and 1cm sampling holes were opened by using sterile knives. Sterile dry sampling swabs were moistened in the rumens, and the swabs were taken to the lab in 2h. The cotton wrapped parts of the 5 swabs were cut by using a sterile scissor and dropped into a flask of 50 ml of alkali (NaOH) solution (pH 10) and left in the alkali for 30 minutes. Then, the content of the flask was acidified until pH 4 by adding %3 citric acid- Na salt solution by continuously stirring on a magnetic stirrer. After a stay in the acid solution for 30 min, the content was neutralized by using 0.1N NaOH, and Phenolphthalein was used as an indicator for pH analysis. A 10ml of the contents was transferred to 100 ml Nutrient Broth, and aerobic incubation was made at 37$^{o}$C for 24h. This medium was used as the stock of inoculum cultures and stored at -18$^{0}$C after portioning and addition of 20% sterile glycerine. The frozen inoculum stocks were thawed at room temperature and grown in Nutrient Broth. Aerobic incubation was made at 37$^{o}$C for 24h. One ml from this fresh broth culture was used as an inoculant for 100 g of some straw samples, as mentioned below.

### 2.3. Samples

For sample preparations, plastic sample boxes at 2 lit volumes were disinfected by using 70% ethanol, and then empty weights with caps were recorded. Seven separate samples (100g straw in each plastic box) were prepared. Alkali water (including 2g NaOH per sample), acidic water (including 3g citric acid-Na salt), inoculant (1ml broth culture) and substrates (1g $KH_2PO4$+ 1g $(NH_4)SO_4$+ 0.3g $CaCl_2$+ 0.2g $MgSO_4$+2g powdered molasses+1g inactive dry yeast) were used separately or in combinations in moistening water. The drinking water was boiled for 1 min and cooled to room temperature before used as moistening water. The NaOH was used in hot water at boiling temperature (97$^{o}$C). The neutralizer, additives, and inoculants were used in the water at room temperature. A total of 500 ml drinking water was added onto each sample as a moistening solution, and after tightly closing the caps on, each box was hand-shaken for 30 seconds to uniformly moist the samples. Alkali treated samples were left at room temperature for 2hrs before other applications. The caps of the boxes were tightly closed to prevent water loss in them during incubations made at 37$^{0}$C for 14 d. Only 2/3 of the sample boxes were full, and handshaking was easily done before taking analysis samples after gently mixing. The analyses were employed on the start day (d=0), 7$^{th}$ day, and 14$^{th}$ day. The experimental design and the sample preparations were summarized in Table 1. The study replicated 3 times, and 2 parallel samples were prepared in each sample series.

### 2.4. Physical and chemical analyses

Water-soluble solids (WSS): After the 14 d incubation, samples were separately weighed, dried, washed 5 times, dried, and the residues of dry matter from each sample were weighed. The % dry

weight was calculated and represented as WSS. Drying was applied at 50°C for 3rd day. As for washing procedure, dried samples in each tight capped plastic box was mixed with 500ml tap water. After gently mixing with a glass rod, water was drained by using a standard tea drainer. The % weight loss from the dry weight of the samples was accepted as micro mass loss and accepted as an indication of the digestion process of lignocellulosic material. The % of the WSS content of the samples was determined by making calculations using the difference between the two dry weights of each sample.

**Table 1:** Sample preparations used in the experiments

| Sample preparations | Content of Moistener | | | | |
|---|---|---|---|---|---|
| 100 g straw was moistened with a total of 500 ml water. The tightly capped boxes were hand-shaken for moistening the contents. The incubations were made under the aerobic condition at 37 °C for 14 d. | Water (ml) | NaOH (2 g) | Citric acid (3 g) | Substrates (5.5 g)* | Inoculant (1 ml) |
| 1 (C): Coldwater (drinking water at room temperature) | 500 | | | | |
| 2 (CI): Cold water+ Inoculant (1 ml rumen culture grown in Nutrient Broth) | 450 | | | | 50 |
| 3 (H): Hot water (drinking water at boiling point) | 500 | | | | |
| 4 (HI): Hot water+ Inoculant | 450 | | | | 50 |
| 5 (HAI): Hot Alkali (2 g NaOH in hot water)+ Inoculant | | 450 | | | 50 |
| 6 (HANI): Hot Alkali + Neutralizer (3 g Citric acid in hot water)+ Inoculant | | 350 | 100 | | 50 |
| 7 (HANSI): Hot Alkali + Neutralizer + Substrates*+ Inoculant | | 300 | 100 | 50 | 50 |

*Substrates (5.5g): $KH_2PO_4$-0.5g; $(NH_4)_2SO_4$- 0.5g; $CaCl_2$-0.3g; $MgSO_4$ -0.2; Molasses-4g

pH: Ten grams from each wet sample was macerated in 90mL of distilled water for 30 min in a shaker and filtered, and the filtrates were used to measure pH with a pH meter (HI 9024; Hanna Instruments Ltd., Leighton Buzzard, UK).

Titratable alkalis/acids (TTA/A): Phenolphthalein in absolute alcohol (1%, w/v) was used as a color indicator. NaOH or citric acid was used as a titration solution. The titration capacity of the solutions was equated by the determination of their neutralization ability to each other, and it was determined that the 1ml of 1.5% citric acid was used for neutralization of 1ml of 1% NaOH. The results were directly given as the ml of 1% NaOH or 1.5% citric acid for neutralization of each liquid sample of 10g of the wet treated sample prepared for pH analysis.

## 2.5. Microbiological analyses

Bacterial counts**:** Tightly closed sample boxes were hand-shaken before analyses made at the 0, 7, and 14th day. Ten g from each sample was transferred to a sterile Erlenmeyer flask, and a 90 ml sterile saline solution (0.9% NaCl) was added on and mixed. One ml from this sample was added to 9 ml of sterile saline solution, and ten-fold serial dilutions were made (Andrews and Hammack, 2003) A 100µL from each dilution tubes was streaked onto 2 parallel agar plates. Plate Count Agar (PCA CM 0325, Oxoid, Basingstoke, UK), was used for counts of total aerobic bacteria (Maturin and Peeler, 2001). The plates were incubated at 32±1°C for 48±3h. Violet red Bile Glucose Agar (VBG CM1082, Oxoid, Basingstoke, UK) was used for counts of *Enterobacteriaceae* (ISO, 2018). Violet red Bile Glucose Agar (VBL CM0968, Oxoid, Basingstoke, UK) was used for counts of coliforms after incubation made at 37°C for 24h (ISO, 2006). Total microbiological counts were expressed as colony-forming units per gram (CFU $g^{-1}$) and were transformed into $\log_{10}$ to obtain the lognormal distribution.

Phenotypical identification: After colony counts, 100 colonies were randomly selected from the plates of each sample and streaked onto NA agar plates for taking pure colonies from them. Purity checking and shape analysis were used during the Gram stain examination. Oxidase and catalase tests were applied to selected Gram+ ve colonies, and then pure colonies grown on NA were used for phenotype analyses by using VITEK 2 Gram-positive kits. A total of 79 colonies were analyzed by using the VITEK 2 Apparatus (Pincus, 2006).

## 2.6. Statistical analysis

All data were analyzed using the general linear model procedure in SAS (2002). Differences among treatment means were determined using the least significant difference (LSD) test. A probability level of $P<0.05$ was considered to be statistically significant.

## 3. Results

At the beginning time of the predigestion process (d 0), the pH of the 5th sample (HAI) was higher than that of the other samples due to the addition of NaOH. The pH of the other samples varied between 6 to 7. The pH of samples increased gradually during the incubations. There was no significant difference from the pH of the samples on the 7th day ($p>0.05$). Also, there was no statistically significant difference from the pH of the first 4 samples between the 7th and 14th day. A statistically significant difference from the pH of the samples 5, 6, and 7 (alkali-treated samples) was determined on the 14th day ($p<0.05$). The samples demonstrated equal pH levels, and there was no statistically significant difference from

the pH of the three samples (p>0.05). These results demonstrate that neutralizer and substrate did not affect the pH of the samples. The positive effect of hot NaOH treatment (samples 5, 6, and 7) on the pH development appeared after 7th d of incubations (p<0.05) (Table 2a).

**Table 2:** The analysis results taken from wheat straw samples treated in different ways after predigestion of the samples incubated aerobically at 37°C

| | 2a-pH | | | 2b- Alkalinity/Acidity (- values) | | | 2c- Water soluble mass (%, dry matter) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0th d | 7th d | 14th d | 0th d | 7th d | 14th d | 0th d | 7th d | 14th d |
| 1. C | 6.6±0.2 | 8.2±0.4[a] | 8.5±0.2[a] | 0.1±0.0 | 0.18±0.01[a] | 0.48±0.02[a] | 2.10±0.3 | 15.36±1.3[a] | 15.43±0.6 |
| 2.H | 6.4±0.1 | 7.9±0.2[a] | 8.4±0.5[a] | 0.1±0.0 | 0.21±0.01[a] | 0.48±0.02[a] | 2.30±0.3 | 14.32±1.8[a] | 18.07±0.8[a] |
| 3.CI | 6.3±0.2 | 8.4±0.3[a] | 8.6±0.4[a] | 0.1±0.0 | 0.18±0.02[a] | 0.63±0.03[a] | 2.10±0.2 | 13.54±1.1[a] | 18.83±1.8[a] |
| 4. HI | 6.3±0.2 | 8.0±0.2[a] | 8.3±0.1[a] | 0.1±0.0 | 0.21±0.01[a] | 0.63±0.02[a] | 2.30±0.2 | 14.13±2.1[a] | 16.47±1.8 |
| 5.HAI | 9.6±0.2 | 9.1±0.1 | 10.1±0.6[b] | 0.9±0.0 | 0.24±0.01[a] | -1.29±0.01[b] | 4.90±0.4 | 20.11±0.9[a] | 20.18±1.3 |
| 6.HANI | 6.1±0.2 | 8.8±0.3[a] | 10.2±0.2[b] | 0.1±0.0 | 0.28±0.02[a] | -1.77±0.0[b] | 5.10±0.6 | 21.26±0.6[a] | 26.58±1.7[b] |
| 7.HANSI | 6.1±0.4 | 8.7±0.1[a] | 10.1±0.3[b] | 0.1±0.0 | 0.42±0.02[a] | -1.70±0.02[b] | 4.90±0.5 | 20.46±1.2[a] | 25.22±1.1[b] |
| | 2d- Total aerobic counts ($log_{10}$ CFU/g) | | | 2e- Enterobacteriaceae counts ($log_{10}$ CFU/g) | | | 2f- Coliform counts ($log_{10}$ CFU/g) | | |
| | 0th d | 7th d | 14th d | 0th d | 7th d | 14th d | 0th d | 7th d | 14th d |
| 1. C | 8.2±1.1 | 8.0±0.8 | 7.3±0.3 | 5.9±1.3 | 5.6±0.7 | 5.3±0.5[a] | 4.5±0.8 | 5.1±0.5 | 3.8±0.3[a] |
| 2.H | 4.9±0.6 | 7.8±0.5 | 8.0±0.5 | 2.3±0.3 | 6.6±0.6 | 4.7±0.4[a] | 2.8±0.6 | 4.3±1.1 | 2.6±0.5[a] |
| 3.CI | 8.5±0.2 | 8.1±0.8 | 8.4±0.4 | 6.1±0.5 | 6.4±0.6 | 4.8±0.4[a] | 4.1±0.2 | 4.7±0.9 | 3.1±0.3[a] |
| 4. HI | 7.8±0.5 | 7.9±0.9 | 8.0±0.8 | 6.3±0.5 | 6.8±0.7 | 3.9±0.3[a] | 3.9±0.3 | 4.4±1.3 | 2.5±0.4[a] |
| 5.HAI | 7.0±0.3 | 8.0±0.4 | 8.8±0.7 | 6.1±0.4 | 6.4±0.5 | 3.3±0.4[a] | 4.1±0.4 | 4.8±0.4 | 2.2±0.2[a] |
| 6.HANI | 7.3±1.1 | 8.3±0.6 | 8.9±0.3 | 6.3±1.1 | 6.5±0.6 | 3.8±0.3[a] | 4.3±0.6 | 4.0±0.4 | 2.3±0.4[a] |
| 7.HANSI | 7.4±0.9 | 8.5±0.7 | 8.9±0.6 | 6.4±0.8 | 6.7±0.9 | 4.7±0.3[a] | 4.0±0.3 | 5.1±0.8 | 2.2±0.2[a] |

[a]: *P*<0.05 versus formerly analysis; [b]: *P*<0.05 versus all the other analysis results
C; cold water, H; hot water, I; inoculant, A; alkali, N; neutralizer, S; substrates added samples

At the beginning of predigestion (0th day), alkalinity in the 5th sample (neutralized by using 0.9ml of 1.5% citric acid) was higher due to the addition of NaOH. The other samples were almost at a neutral level. At the 7th d, alkalis of all samples were higher than that of the 0th day. The high alkali level of Sample 5 decreased to the level of the other samples. All the samples demonstrated alkali contents in them. The 7th sample demonstrated more alkali level than others at the 7th d of fermentation. At the 14th d of fermentation, alkali contents of the first 4 samples increased significantly (P<0.05). Contrary to the first 4 samples, samples 5, 6, and 7 were acidic at the d 14. The results demonstrate that hot alkali treatment had a positive effect on the acid development in the samples at the 14 d (P<0.05). Acids levels of the samples 5, 6, and 7 at the 14th d were 1.29 (ml of 1.5% citric acid) 1.77 and 1.70, respectively. These results demonstrate a positive effect of neutralization on acid development (P<0.05), and no positive effect of substrate addition on it (P<0.05) (Table 2b).

At the d 0, weight loss after washing the samples was about 3 to 5%. The effect of alkali treatment was about 2% at the d 0, and no difference between neutralizer and substrate treatment was seen. At the d 7, weight loss from the first 4 samples was between 13.5 % and 15.3%, and there was no significant difference among these samples (p>0.05). Nevertheless, weight loss from the last 3 samples was significantly higher than that of the first 4 samples (P<0.05). Weight loss from all the samples except for the 1st sample increased between the 7th d and 14th d of incubation. The highest weight loss at the 14th d was seen in the 6th and 7th samples when compared to that of others (P<0.5). These results demonstrate that predigestion in the samples increased significantly when neutralization was applied, while no positive effect of the addition of supplements was seen (Table 2c).

The total aerobic microflora of the samples decreased by about 3 log units when hot water was applied (P<0.05) (Table 2d). The culture addition elevated the numbers of total aerobic bacteria of the hot water treated samples at the d 0 (Samples 4, 5, 6, and 7) (P<0.05). There was no statistically significant difference among the total bacterial load of the samples at the 7th d (p>0.05). On the 14th day, a hot alkali-treated sample (Samples 5-7) had a higher bacterial load than that of hot alkali untreated samples (Samples 1-4) (P<0.05). There was no difference from total aerobic bacterial load among the samples that hot alkali (Sample 5), neutralizer (Sample 6), or neutralizer with the substrate (Sample 7) added (Table 2a) (P>0.05).

The *Enterobacteriaceae* population of the samples decreased by about 4 log units when hot water was applied (Table 2e). The culture addition elevated the reduced numbers of *Enterobacteriaceae* of the samples at the d 0. There was no statistically significant difference between *Enterobacteriaceae* counts of the samples at 7th d. At the d 14, *Enterobacteriaceae* counts in all the samples decreased significantly (P<0.05). Sample 5, in which neither neutralizer nor substrate was added, appeared to be the hygienic sample among the other 6 samples. Hot alkali treatment appeared more effective on the hygienic quality of the wheat straw after 14 d of incubation at 37°C by the addition of a ruminal semi-selective microbial inoculant.

The total numbers of coliform bacteria in the samples decreased when the hot alkali-treated (Table 2f). At the d 7, an increment of about 1 log unit from total coliform counts in the samples was detected. The coliform counts from each sample decreased about 2 log units at the d 14. This

demonstrates that the hygiene of the samples became more reasonable at the 14th d of incubation. While a positive effect of alkali treatment was seen, no positive effect of neutralization and substrate addition demonstrated at the 14th d. Hot alkali treatment had low coliform levels at the 14th d of incubation ($P<0.05$).

The VITEK GP Test results of 43 identified strains among 79 Gram +ve aerobic strains selected from wheat straw samples predigested aerobically at 37°C for 14 d are represented in Table 2. Among them, 12 *Staphylococcus lentus,* 8 *Dermococcus* and *Kytococcus,* 4 *Staphylococcus haemolyticus,* 4 *Kocuria kristinae,* 3 *Enterococcus faecium,* 2 *Enterococcus columbae,* 2 *Globicatella sanguinis,* and 10 other strains were determined. A total of 43 tests were applied to each of 79 strains. The test results of all strains are demonstrated in Table 3.

**Table 3:** The VITEK GP Test results of 43 identified strains among 79 Gram+ ve aerobic strains selected from wheat straw samples predigested aerobically at 37 oC for 14 d

| No.* | Identification (ID) results | ID (%) |
|---|---|---|
| 12 | *Staphylococcus lentus* | 89-97 |
| 8 | *Dermococcus* and *Kytococcus* | S,E,G.** |
| 4 | *Staphylococcus haemolyticus* | 94 |
| 4 | *Kocuria kristinae* | 85-93 |
| 3 | *Enterococcus faecium* | 85-94 |
| 2 | *Enterococcus columbae* | 95-99 |
| 2 | *Globicatella sanguinis* | 91-94 |
| 1 | *Enterococcus faecalis* | 95 |
| 1 | *Streptococcus thoraltensis* | 89 |
| 1 | *Gemella sanguinis* | 86 |
| 1 | *Enterococcus casseliflavus* | 90 |
| 1 | *Alloiococcus otitis* | 89 |
| 1 | *Kocuria varians* | 87 |
| 1 | *Dermococcus, Kocuria* | LD*** |
| 1 | *Enterococcus faecalis or Enterococcus faecium* | LD |
| 1 | *Gemella or Leuconostoc* | LD |
| 1 | *Enterococcus casseliflavus, Enterococcus faecium* or *Enterococcus gallinarum* | LD |

*: Numbers of the strains isolated, **: Slimeline, excellent, good; ***: Low discrimination

## 4. Discussion

For predigestion, harsh acidic and alkali treatments with moistening water at high temperatures have been applied in many studies (Imman et al., 2018). Liquid hot water at elevated temperature (170–230°C) and pressure (up to 5 MPa) is used as a moistener in place of steam (Boakye-Boaten et al., 2015). Imman et al. (2015) demonstrated that rice straw pretreated with liquid hot water in the presence of NaOH showed remarkably higher glucose yield compared with liquid hot water pretreatment in the absence of NaOH. Likewise, the presence of acid and alkali promoters changes the physical structure of the pretreated biomass (Imman et al., 2014). Nevertheless, for the preparation of wheat straw as predigested feed material, these harsh applications could not be applied at the farm level in rural areas. Also, it may not be economically affordable, and safety requirements could not be taken. Rural areas have plenty of solar energy for heating the moistening water, but it doesn't have cheaper energy sources for steam production. We used hot tap water for moistening, which can be produced in rural areas by using solar energy source during hot climatic seasons. The use of steam moistening appears not to be feasible for such a purpose. Thus, in this study, we used a low level of NaOH (2% of the dry weight of the straw) in hot water (0.4% of the water) to increase predigestion. This level of NAOH may be safe for application and economically feasible for under-developed rural areas. Predigestion process used for increasing the possibility of ruminal digestion quality, and accordingly, economic animal breeding appeared to be safely applicable, economically feasible. Nevertheless, more studies, including feeding trials, have to be conducted on the subject.

After the physical and chemical process, one or few bacterial, yeast+bacterial, or mold strains that are not from rumen origin have been used for predigestion of the samples straw with different time periods (Jiménez et al., 2014). Ruminal inoculants have also been used for such purposes (Gado et al., 2017; Zhang et al., 2017). Cow manure has also been used directly for predigestion of straw (Hassan et al., 2011). We could not come across to a predigestion study, in which aerobic rumen microflora that is resistant to pH 10 and pH 4 for 30 min has been used. We used such a culture in this study. So, we expected that the culture could have grown more successfully in the samples treated with alkali and/or acids before the microbial predigestion period. Natural microflora of wheat samples was not excluded from the samples, and that natural microflora also acted with our intentionally added rumen flor in the experiments. The un-documented sensorial findings of this study have demonstrated that samples 1 to 4 (C, H, CI, and HI) had manure odor and color after 7th d of incubations, and also mold development at high rates. The samples were clearly seen as composted, and thus they could not be accepted as feed materials. Nevertheless, samples 5 to 7 (HAI, HANI, and HANSI) had better color and odor and appeared to be in the acceptance rates as for feed materials. Not a visible mold growth was seen in these samples. The results can be taken into consideration as preliminary findings for further studies. More specific studies have to be carried out on these subjects. We could not clearly point out the net usefulness of inoculation culture (Table 2). This may possibly be due to the presence of natural straw flora that is resistant to predigestion applications used in the study. The competition between the two microflora has also not been analyzed in this study. More useful, an inoculation culture for such purpose can be determined in further studies.

Straw particle size and mixing treatments also have an important effect on the predigestion process (Gallegos et al., 2017). In this study, we did not make particle size reduction from the size at 2 to 5 cm that preferred for cow feed since the aim was to make more digestible a feed material from wheat straw. Therefore, the same feed material could afford the

ballast material that is needed for rumination health. Mixing was not applied to the samples because our aim was to search for an easy way of predigestion, which can be a suitable application for small farm environments.

The hygienic status of feeds is under strict governmental regulations. Counts of total aerobes are accepted as hygiene criteria in the regulations of the food and feed materials. Nevertheless, the population of aerobes determined in this study was not informative due to the addition of inoculation culture and prolonged fermentation (Table 2d). The family *Enterobacteriaceae* encompasses 30 established genera, including *Salmonella spp*, Escherichia spp, Shigella spp, and Yersinia spp. So, *Enterobacteriaceae* contamination level in feedstuff is accepted as an indicator of feed hygiene. In a previous study, a large proportion of the raw materials used for animal feed manufacture were contaminated with significant levels of *Enterobacteriaceae* (>$10^4$ CFU/g). Processing the raw material has the potential to reduce this degree of contamination. The results of this study demonstrate that counts of *Enterobacteriaceae* and coliform group bacteria at the 7$^{th}$ d of incubation was not at the aimed levels. Nevertheless, at the 14$^{th}$ d of incubation, the values appeared to be more reasonable in the last 3 samples, but these reasonable results were only determined in the last 3 samples (HAI, HANI, and HANSI) in which hot alkali, neutralizer, and inoculant were used. No reasonable result was determined in the first 4 samples, in which cold or hot water with inoculant were used. Low levels of *Enterobacteriaceae* (Table 2e) and Coliforms (Table 2f) were in accordance with the development of the acidic environment in sample 5 (HAI), in which no neutralizer or substrate was used. When these results were compared with the results of the samples 6 (HANI) and 7 (HANSI), it is understood that there is no need for the addition of extra chemicals such as neutralizer or substrates except for NaOH. The results demonstrate the prevention of extra cost and effort. Nevertheless, more studies have to be made for developing a hygiene promoting inoculation culture for microbial wheat straw predigestion.

In a previous study, straw samples were pasteurized before preparing sample materials (Fazaeli et al., 2004). Industrial predigestion applications can be suitable for washing or pasteurizing the straw before use. Nevertheless, washing or pasteurizing may not be feasible economically for rural areas, and water sources may also not be appropriate for such applications. We did not wash the straw samples before treatments. Since the natural flora enters the rumen of animals with ingested straw, the predigestion process was also applied to the un-washed straw samples in that study, so natural microflora of the straw samples prepared for feeding cows acted in the microbial process. In such studies, more detailed tests have to be done to determine the interaction between the natural straw flora and inoculation culture.

The activities of bacteria may be controlled to facilitate the preservation of ensiled forages. Additives may be used to control or stimulate fermentation by bacteria in ensiled forages. Formic acid is used as for fermentation restriction agent by artificially reducing pH to below 4.0. The addition of urea, molasses, lime, and some other substrates with microbial inoculants to the ensiled forages brings out hygiene by making the desired reduction in pH values. The efficacy of this procedure depends upon the inoculation rate as well as the presence of adequate concentrations of sugars (D'Mello, 2004). These subjects are well studied for making hygienic silage from raw forages, and many preparations are available at commerce. Nevertheless, both additives and inoculation cultures have not yet been commercially recommended for the predigestion of straw. Pathogenic bacterial growths and/or toxins present in feeds of animals have demonstrated to be important for animal and public health (Alali and Ricke, 2012). This situation appears to be more important when microbial predigestion was applied to feed material. In this study, we did not pasteurize or sterilize the straw samples before inoculations, and thus some possible pathogenic bacteria could have also grown in the samples. Furthermore, although we treated the inoculation culture in alkali and acidic conditions, some infectious, toxigenic, and/or toxic/infectious strains could have remained in the culture. Among the 43 isolates, 12 of *Staphylococcus lentus,* 8 of *Dermococcus* and *Kytococcus,* 4 of *Staphylococcus haemolyticus,* 4 of *Kocuria kristinae,* 3 of *Enterococcus faecium,* 2 of *Enterococcus columbae,* 2 of *Globicatella sanguinis,* and 10 of other separate strains were determined (Table 3). Some of these strains have been demonstrated as a pathogen for humans when each strain was searched from the Pubmed. This is important because the strains are isolated among the agar plates of the highest serial dilutions of the samples. This means that the identified strains were among the most dominant microflora in the samples. Also, straw samples could have also had some naturally occurring pathogenic or toxin-producing microorganisms. Another important result is that there was not a correlation between routine hygiene indicators (counts of *Enterobacteriaceae* and coliforms) and the 43 isolates. The hygienic status became better in the samples on the 14$^{th}$ d of predigestion. Nevertheless, some pathogenic strains determined from the dominant flora of the samples appeared to have infectious character. The results have to be taken into consideration to prepare hygienic predigested feed material. Also, hygiene regulations for biological pretreated feed materials may be evaluated in this point of view, and rumen dependant animal health has to be evaluated under the light of new scientific achievements.

## 5. Conclusion

Predigestion of wheat studies was mostly made by using fungi. Also, limited strains separately or in

combinations have been used. There are limited studies conducted on the use of complete or partial bacterial consortia of rumen flora in such purposes. We preferred to in this study alkali and acid-stable consortia of bacteria of the rumen. Also, we aimed to determine possible sporadic pathogen strained from the predigestion media of wheat straw. The study can be accepted as a different look at prefermentation process. Addition of a 2g NaOH as alkalizer with moistening hot water and a 3g citric acid as neutraliser appeared to be effective on the prefermantation process of 100g f wheat sample. Additional supplements such as $KH_2PO_4$, $(NH_4)SO_4$, $CaCl_2$, $MgSO_4$, molasses and inactive yeast appeared to be no needed in such application. Nevertheless, some possible pathogenic bacteria were dominately grown in the process. These finding may be informative for further studies conducted on hygiene requirements of such studies. Selection of microbial flora from rumen content, and successive and healthy predigestion process parameters could be extensively investigated in the process of predigestion of wheat straw used as feed material.

## Acknowledgment

The authors appreciate the research support provided by a grant from the Excellence Center of Near East University, Nicosia/Cyprus (Grant no. SAG-2016-04047).

## Compliance with ethical standards

## Conflict of interest

The authors declare that they have no conflict of interest.

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