In vitro methods to evaluate the effects of plant waste products on rumen and gut microflora

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ABSTRACT: The requirement of a safety use of additives in animal nutrition induced the EU to introduce a new regulation on feed additives. Organic wastes from the food/feed industry could include valuable substances to novel high value added products as additives. The objective of the present study was to screen in vitro effects of organic wastes on rumen microbial metabolism by batch incubator system (pH, ammonia N, VFA concentration, Total Bacterial Count) and on gut microbial flora from monogastrics by paper disc agar diffusion method (growth or inhibition of gut microbial flora). The results obtained by these preliminary in vitro tests have permitted to identify the substances with positive activities that will be evaluated in future in vivo trials.

Key words: Rumen, Gut microflora, Waste products, Additives.

INTRODUCTION – Gastrointestinal tract (GIT) fermentations have an important influence on health both of monogastrics and ruminants. The fermentation process is determined by many factors including the host, its microflora and the interactions between them. The stressful events in critical periods (e.g. transition period in dairy cow and weaning in piglets) shift microbial populations toward an increase in pathogenic organisms that can alter the fermentation process and have negative effect on health of the GIT itself and also of the host animal. For this reason, feed antibiotics and chemotherapeutics have been extensively added to animal nutrition to modulate microbial species. The EU focused on effective alternatives to reduce the increasing number of antimicrobial-resistant bacteria strains with the aim to evaluate new potential additives safe for animal and human nutrition. The industrial processing of fruits, vegetables and the extraction of phytotherapeutic compounds from plants, produce many tons of organic waste that could include valuable compounds (e.g. polyphenoles, flavonoids) that could be further utilised with a specific efficacy on animals (Tedesco et al., 2007). The SAFEWASTES project (EU project n. 513949) focuses on the research and development of new methods to transform wastes from the food industry and phytotherapeutic (not considered harmful for human or animal health), into high added-value products. The aims of the present study were to screen the in vitro effects of different waste products 1) on the rumen microbial fermentation, by batch incubator system; 2) on naturally occurring gut microflora of monogastrics, by in vitro paper disc agar diffusion method.

MATERIAL AND METHODS – Specific post-processing derivative waste products from the food industry and the plant-based extract industry were evaluated as raw material (aerial parts of the plant chuffed, pressed and dried; roots washed, blanched, crushed, pressed and dried; fruits, pulp of fruit and peels crushed, dried and powdered; whole seeds heated, extracted, crushed and powdered) and after extraction with water, ethanol and heptan. In this study, a total of 52 waste products were tested. 1) Rumen trial: The in vitro batch culture incubation system was carried out with mixed rumen fluid withdrawn from three rumen-fistulated not-lactating dairy cows. The rumen fluid was added to a mineral salt buffer, prepared as described by Op den Camp et al. (1988). The rumen fluid and the mineral salt buffer were mixed in a bottle warmed at 39°C. The batch culture of rumen fluid was purged whit anaerobic grade N2/CO2 (80/20,v/v) and standardized at pH 6.8 ± 0.1 with NaOH. 100 mL of solution were placed in glass bottles supplied whit 0.8 g alfalfa hay and 0.2 g corn meal (as a substrate for microbial growth).
The tested substances were added to the bottles at three different concentrations: raw material at 1.2, 2.4 and 12 g/100 mL; ethanol, heptan and water extract at 0.05, 0.1 and 0.5 mg/mL. Each test was evaluated in duplicate. The bottles were purged with anaerobic grade N2/CO2 (80/20, v/v), closed, and incubated in a water shaking bath at 39°C for 24 h. Samples were withdrawn after 0, 4, 9, and 24 h and analysed for pH, VFA, NH3 and TBC (total bacterial count). The pH was determined at 0, 4, 9, 24h of incubation using a pH-meter. At 0 and 9h of incubation the production of VFA (acetate, propionate, butyrate and isobutyric acid) was determined by gas chromatograph (GC Clarus 500, Perkin Elmer Inc., Shelton, CT, USA), equipped with a column packed (Superchrom, Milano, Italy). The ammonia N was determined at 0 and 9h of incubation by Kjeldhal method. The TBC (TSA, VWR-Merck) was evaluated at 0h and after 24h of incubation.

2) Gut trial. Faecal samples from rectal ampulla of healthy piglets (60–70 days of age) were taken by sterile spatula. The mixed samples were introduced in a stomacher bag and diluted 1:10 (v/v) with 0.9% NaCl/tryptone solution. Decimal dilutions were prepared and 1mL aliquots were inoculated in Petri dishes with specific solid culture media to promote the bacterial growth. Five strains of *E. coli* (Chromocult agar, VWR-Merck), Clostridia (TSC agar, VWR-Merck) and Lactobacilli (MRS agar, VWR-Merck) were isolated. Aliquots of 0.1 mL of bacterial suspension of each selected strain (10⁸ CFU/mL) were inoculated onto the surface of agar plates containing the specific culture media. The effects of the tested substances, on the naturally occurring microflora, have been studied using the paper disc agar diffusion method (Kirby-Bauer Method). 0.5 g of each water extract was solubilised into deionized water (50ml), while ethanol extract 0.5 g aliquots were solubilized into 50 mL of absolute ethanol. The solutions obtained were sterilized by filtration and put into sterile tubes. Sterile paper discs (Oxoid) were moistened with sample solutions and dried for 30 min. into a laminar flow hood before use. 1) a test paper disc moistened with the extract; 2) a positive control (a disc containing 10 µg of Ampicillin for Clostridia and Lactobacilli and a disc containing 15 µg of Apramycin for *E. coli*); 3) and a negative control (a disc moistened with sterile deionised water or absolute ethanol) were placed under aseptic conditions onto the surface of inoculated culture medium. The plates were maintained at room temperature for 2 h allowing the diffusion of the solution, then incubated at 37°C for 24 h, in aerobic (*E. coli*)/anaerobic (Lactobacilli, Clostridia) conditions. After incubation, plates were observed in order to find the presence of growth inhibition areas and measure them.

**RESULTS AND CONCLUSIONS** – No negative effects were evidenced on rumen microbial fermentation and on gut microbial population, following the incubation with the tested substances. These preliminary results can suggest that these substances could be potentially used as additives if other positive physiological functions will be evidenced (e.g. as anti inflammatory, immunostimulant).

**Table 1.** SAFEWASTES by-products test: effects on rumen fermentation.

| N test (1-52) | Substances | Processing | pH | NH₃ | VFA |
|--------------|------------|------------|-----|-----|-----|
| 5            | TV         | Raw material | ↓   | -   | -   |
| 13           | SA         | Raw material | ↓   | -   | -   |
| 17           | CF         | Raw material | ↓   | -   | -   |
| 21           | SI         | Raw material | ↓   | -   | ↓   |
| 31           | MI         | Ethanol extract | ↓   | ↓   | ↑↑  |
| 34           | VV         | Water extract | ↓   | -   | -   |
| 35           | VV         | Ethanol extract | ↓   | -   | -   |
| 36           | VV         | Heptan extract | ↓   | -   | -   |
| 39           | SR         | Ethanol extract | ↓   | -   | -   |
| 40           | SR         | Heptan extract | ↓   | -   | -   |
| 41           | LD         | Raw material | -   | ↓↓  | ↑↑  |
| 42           | LD         | Water extract | -   | ↓↓  | ↑↑  |
| 45           | DC         | Raw material | -   | ↑↑  | ↑↑  |
| 46           | DC         | Water extract | -   | ↑↑  | ↑↑  |
| 47           | DC         | Ethanol extract | -   | ↑↑  | ↑↑  |
| 50           | CP         | Water extract | ↓   | ↑↑  | -   |

↓↓: decrease value >10% vs control; ↓: decrease value from 5% to 10% vs control; ↑↑: increase value >10% vs control; ↑: increase value from 5% to 10% vs control; -: no significative effects.
Table 2: SAFEWASTES by-products test: effects on gut microflora.

| N test | Substance | Processing | E. coli | Lactobacilli | Clostridia |
|--------|-----------|------------|---------|--------------|------------|
| 46     | DC        | Water extract | +/-     | -            | -          |
| 47     | DC        | Ethanol extract | -       | -            | +/-        |
| 2      | CS        | Water extract | -       | -            | +/-        |
| 3      | CS        | Ethanol extract | -       | -            | +/-        |
| 22     | SI        | Water extract | +/-     | -            | -          |
| 18     | CF        | Water extract | +/-     | +/-          | -          |
| 50     | CP        | Water extract | -       | +/-          | +/-        |
| 26     | AH        | Water extract | +/-     | -            | -          |
| 42     | LD        | Water extract | +/-     | -            | -          |

+/− = slight halo of inhibition; − = absence of halo of inhibition.

Rumen trial: The TBC value after 24h of incubation proved the stability of the in vitro batch incubations system. A small decrease in pH value, within physiological ranges, after inclusion of some of the products suggests an effect on ruminal fermentation. Among the 52 tested substances, 25% influenced N and/or VFA parameters indicating an effect on ammonia N relative to control. The reduction of ammonia N concentration can be probably due to an improved efficiency of N utilisation (Busquet et al., 2006). The VFA are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants. It is interesting to point out that the results obtained from 5 tested samples increased the total VFA concentration compared with control. Two of the tested samples evidenced both positive effects: a decreased ammonia N production associated to an increased VFA production. Careful selection and combination of these extracts may improve the efficiency of N utilisation and energy in the rumen and may allow the manipulation of rumen microbial fermentation. As reported in table 2, the study on gut microbial flora from piglets evidenced that 4 extracts showed an inhibitory effect on E. coli and coliform growth; 3 extracts showed a small inhibitory effect on Clostridia growth; one tested substance evidenced a very slight inhibitory effect on Clostridia and Lactobacilli. These preliminary in vitro results permitted to identify the substances with positive activities that will be further evaluated with in vivo trials to confirm the results obtained.

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REFERENCES - Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2006. Plant extracts affect in vitro rumen microbial fermentation. Journal of Dairy Science, 89(2): 761-771. Op den Camp, H.J.M., Verhagen, F.J.M., Kivaisi, A.K., De Windt, F.E., Lubberding, H.J., Gijzen, H.J., Vogels, G.D., 1988. Effects of lignin on the anaerobic degradation of (ligno) cellulosic wastes by ruminal microorganisms. Applied Microbiology and Biotechnology, 29: 408-412. Tedesco, D., Stella, S., Garavaglia, L., Barbieri, C., SAFEWASTES by–products and functional effects on gut colonic microbiota epithelia-associated. 21st Meeting of the European Intestinal Transport Group held in Oberwiesenthal, March 03-06, 2007.