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Role of Herbal Residues in Pathogen Inhibition and VFA Production by *in Vitro* Studies in Pigs

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ABSTRACT

A basal diet was added with six herbal residues viz- Bacopa monnieri Withania sominifera, Garcinia cambogia, Gingeber officinale, Emblica officinalis, Curcuma longa to make six dietary treatments (T_1-T_6),respectively to evaluate volatile fatty acid production and their ability to inhibit pathogen growth in a CRD model. It was observed that the lowest ($P<0.05$) pathogenic count was recorded for T_4 as compared to others. In T_1 to T_6, acetic acid production non-significantly dominated propionic acid followed by butyric acid. It was concluded that herbal residues especially Zingiber officinale can be hypothetically used as an alternate to antibiotics in pigs in improving the performance indices.

Key words: In vitro, herbal residues, Volatile fatty acid production, pathogen inhibition

1. INTRODUCTION

Like in ruminants the *invitro* fermentation method gained importance in monogastric animals also. For the purposes of food evaluation, in vitro digestion/fermentation methods are ethically superior, faster and less expensive than *in vivo* techniques. The large intestines provide a chamber for the final phase of digestion in pigs where it involves the breakdown of carbohydrates releasing short chain fatty acids (SCFA) predominantly acetate, propionate and butyrate (Cummings and Macfarlane, 1991) with traces of isobutyrate and mixture of gases (H_2, CO_2 ,CH_4). Various factors like type and chemical nature of polysaccharides fermented, activities of the colonic microbial population and transit time in the GI tract affect the composition and molar proportions of the SCFA production in the lower gut (Englyst *et al.*, 1987). During the last few years fermentation in the lower gut gained importance for two fold reasons –the

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SCFA produced regulates the intestinal micro-organisms and they also contribute energy to a tune of 15% of maintenance needs in growing-finishing pigs (Dierick *et al*., 1989) and 30% for gestating sows (Varel and Yen, 1997). The pig diet after hydrolyzing with the enzymes pepsin and pancreatin in the lab (Boisen and Fernandez, 1997) is incubated anaerobically by adding pig faecal inoculum as a bacterial source.Public concern over use of antibiotic feed additives has lead to research on alternative substances like herbal residues with antimicrobial properties. It was reported that supplementation of phytogenic feed additives when compared with antibiotics or organic acids had similar effects on the gut in pigs and poultry (Windisch *et al*., 2008).

The present experiment was planned with the aim to study the role of herbal residues on pathogen inhibition and VFA production for the enzyme hydrolysed feeds incubated with pig faecal inoculum (Bindelle *et al*., 2007)

2. MATERIALS AND METHODS

2.1. Animals and diet

The faecal inoculum was prepared from twelve 75% Large White Yorkshire cross-bred female pigs (24.3± 1.10 kg).The animals were fed (NRC,1998) *adlibitum* and were kept in groups with free access to water. The collection of faeces was started when the animals were adapted to the feed for over 4 weeks.

2.2. Dietary treatments

A basal diet (NRC, 1998) was treated with Pepsin followed by Pancreatin enzymes (Boisen and Fernandez, 1997). The enzyme hydrolysed dried residue was added with six herbal residues viz- *Bacopa monnieri, Withania sominifera*, *Garcinia cambogia*, *Zingiber officinale*, *Emblica officinalis*, C*urcuma longa* to form six treatments (T_1 to T_6).These dietary treatments were incubated with faecal inoculum in quadruplicate to study the fermentation pattern. The diets (basal and hydrolyzed) were analyzed (Table.1) for proximate composition (AOAC, 1995). Data was subjected to One-way analysis (Snedecor and Cochran, 1989)

2.3. Minimum Inhibitory Concentration test

The disc diffusion method was used to determine the antimicrobial activity of the herbal residues. All the herbal residues were diluted in diethyl ether from 0.2% to 2.0%. The sensitivity of the individual herbal residue was classified by the diameter of the inhibition zone (Moreira *et al.*, 2005). Agar diffusion assay (Moreira *et al.*, 2005) was used to determine minimum inhibitory concentration (Table.2) of the herbal residues and after the incubation at 37^0C for 24 hours, the inhibition zones were measured.

2.4. Pepsin-Pancreatin hydrolysis

Enzymatic hydrolysis was done in 2 batches with 10 replicates. An amount of 90.4 g (9.0 g, 10 replicates) and 89.6 g (8.9g, 10 replicates) of basal diet was taken for enzymatic hydrolysis for batches one and two, respectively.

The basal diet residue after pepsin-pancreatin enzyme hydrolysis (Boisen and Fernandez,1997) was collected into previously weighed crucibles and dried in hot air oven at 100 ^{0}C ± 0.5 for 6 hours, cooled in the desiccator and weighed. The difference of weights of crucible with dried residue and empty crucible was calculated.

2.5. Preparation of inoculum

Buffer solution was prepared (Menke and Steingass, 1988) and warmed at 37 ^{0}C until faeces was added. The faeces for bacterial source was collected from twelve 75%Large White Yorkshire cross-bred female pigs (24.3± 1.10 kg) directly in four CO_2 fluxed 100 ml plastic sterile containers (since the dietary treatments were incubated in quadruplicate, three animals were selected for each replicate) and were immediately placed in a water bath at 39 ^{0}C (Bindelle *et al.*, 2007) for transportation to the laboratory. In order to reduce the variation between animals, about 28 g faeces were collected from three pigs for bacterial source for each replicate. Faeces were used as the inoculum since the faecal microflora can be considered as representative of the large intestinal microflora (Coates *et al.,* 1988; Williams *et al.*, 1998).

About 210 ml pre-heated (39 ^{0}C) buffer medium was added to each of the plastic containers containing faecal samples. All the samples were subjected to mechanical pummelling using an ordinary laboratory blender for 60 seconds in order to suspend fibre-associated bacteria in the liquid (Merry and MacAllan, 1983). Then the solution is filtered through a 250 µm mesh screen and the filtered solution was made up with 1.5 litre buffer medium (Bindelle *et al*., 2007) in order to reach a dilution of 0.05 g faeces per ml buffer. During the entire process care was taken to maintain anaerobiasis by continuous bubbling with CO_2.

After centrifugation of the fermented contents one ml of the supernant liquid was collected into sterile 2 ml plastic containers already added with 0.2 ml of 25% Metaphosphoric acid and were preserved for VFA estimation at -20^{0}C. Volatile fatty acids were estimated using CERES 800 plus series gas chromatography. The total bacterial load (CFU/ml) was counted in the fermented contents at the end of the fermentation (Fig.2) to evaluate the efficacy of the herbal residues in preventing the growth of pathogenic bacteria.

3. RESULTS AND DISCUSSION

3.1. Bacterial load

It was observed that the lowest pathogenic count (total bacterial count, Coliform, *Salmonella* and *Staphylococcus*) was recorded (Table.3) for T_4 as compared to others. It was shown that *Zingiberis* residue was effective in inhibiting the growth of pathogens. A control was run for all replicates and it was observed that the bacterial load was higher ($P<0.05$) as compared to other treatments. In the present experiment it was observed that herbal residues are able to check the growth of bacteria during fermentation. Higher OM fermentation, higher acetic acid production, lower pH could be the probable reasons for a lower bacterial count in T_4, since these factors can arrest the growth of undesirable bacteria especially *Salmonella*. It is well known that the presence of the SCFA will lead to a drop in pH that can have a negative effect on some potentially pathogenic bacteria (Williams *et al*., 2005). It has also been shown that SCFA inhibit the growth of *Salmonella* (Van derwielen, 2001). VFA

can have an antibacterial effect, thereby preventing the establishment of pathogenic bacteria, such as *Salmonella* spp. (Cummings and Englyst, 1987).

Fig.1: Effect of treatment diets on Pathogen inhibition

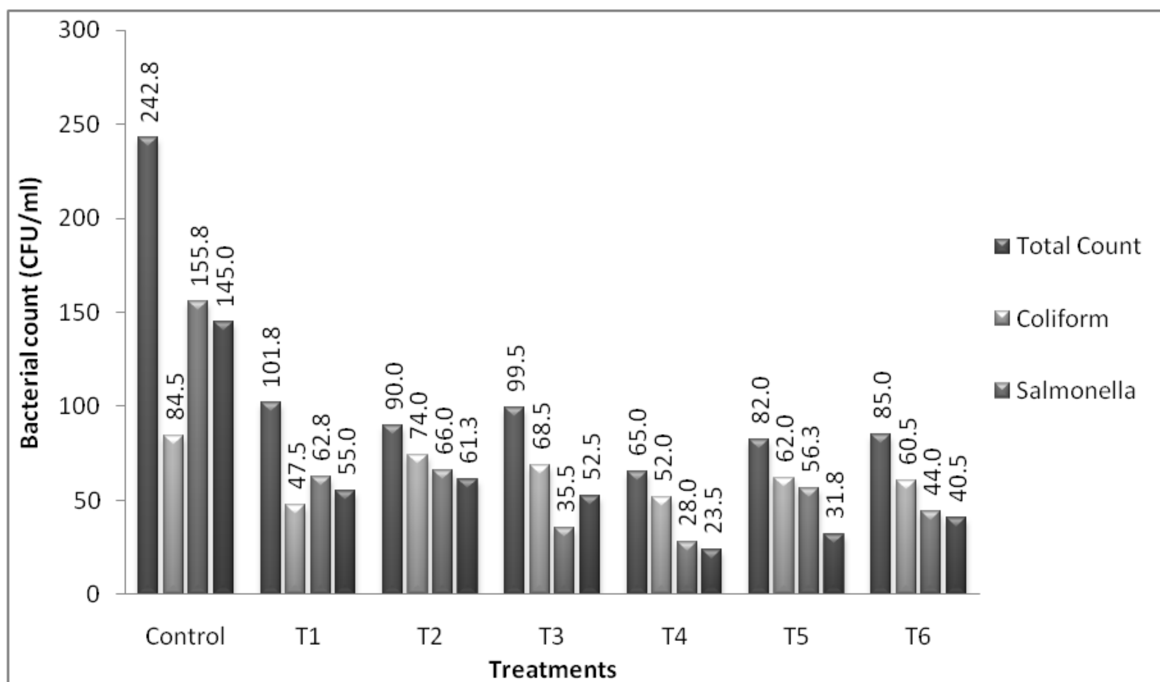

Fig.2: Effect of treatment diets on Volatile Fatty Acid Profile.

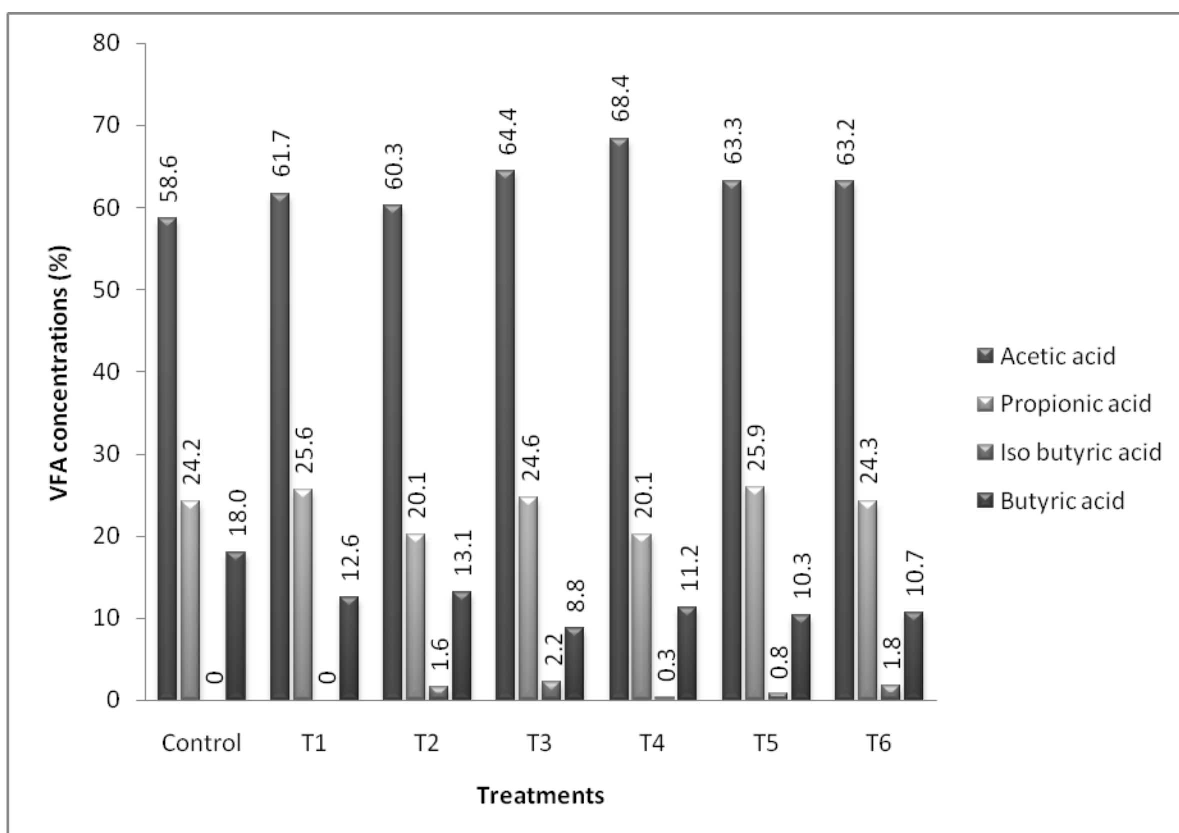

Table 1. Analysis of the basal and enzyme hydrolyzed diets

Nutrient (%)	Basal diet	Hydrolysed diet
Dry Matter	90.3	91.2
Organic Matter	87.2	85.1
Crude Protein	15.7	6.3
Ether Extract	1.8	0.82
Crude Fibre	9.3	13.4
Total Ash	12.7	14.8
Nitrogen Free extract	60.3	64.6
Neutral Detergent Fibre	63.6	68.8
Acid Detergent Fibre	22.8	27.4

Table 2: Effect of herbal residues on pathogen inhibition(Moreira et al., 2005)

Concentration of herbal residue (%)

Name of the bacteria *	*Bacopa monnieri*							*Withania sominifera*						
	0.2	0.4	0.6	0.8	1	1.5	2	0.2	0.4	0.6	0.8	1	1.5	2
a	-	-	+	+	+	+	++	-	-	-	+	+	++	++
b	-	-	-	+	+	++	++	-	+	-	+	+	+	++
c	-	+	-	+	+	++	++	-	-	-	+	+	++	++
d	-	-	-	+	+	+	+++	-	-	-	-	+	+	++
e	+	-	-	-	-	+	++	-	-	+	-	+	++	++
f	-	-	+	-	+	+	++	-	-	+	-	+	+	++
g	-	-	+	-	+	+	+++	-	-	-	-	+	+	++
h	-	-	-	-	+	+	++	-	-	-	-	+	-	++

Name of the bacteria *	*Garcinia cambogia*							*Gingeber officinale*						
	0.2	0.4	0.6	0.8	1	1.5	2	0.2	0.4	0.6	0.8	1	1.5	2
a	-	-	-	+	+	+	++	-	-	-	+	+	+	++
b	-	-	-	+	+	+	+	-	+	-	+	+	+	++
c	-	-	+	+	+	++	++	-	+	-	+	+	+	+
d	-	-	-	-	+	+	+	-	-	-	+	+	+	++
e	-	-	-	-	+	++	+	+	-	-	-	-	+	++
f	-	-	+	-	+	++	++	-	-	+	-	+	++	+++
g	-	-	-	-	+	+	++	-	-	+	-	+	+	+++
h	-	-	-	-	+	+	++	-	-	-	-	+	+	+++

Name of the bacteria *	*Emblica officinalis*							*Curcuma longa*						
	0.2	0.4	0.6	0.8	1	1.5	2	0.2	0.4	0.6	0.8	1	1.5	2
a	-	-	-	+	+	-	+	-	-	-	+	+	+	++
b	-	-	-	+	+	+	+	-	+	-	+	+	+	++
c	-	-	-	+	+	+	+	-	+	-	+	+	+	+
d	-	-	-	-	+	+	++	-	-	-	+	+	+	++
e	-	-	-	-	+	+	+	+	-	-	-	-	+	++
f	-	-	-	-	+	++	+	-	-	+	-	+	++	++
g	-	-	+	-	+	+	++	-	-	+	-	+	+	++
h	-	-	-	-	+	-	++	-	-	-	-	+	+	++

*** a= *Escherichia coli;* b= *Staphylococcus aureus;* c= *Salmonella typhimurium;* d= *Bacillus cereus jejuni;***
e= *Campylobacter;* f = *Listeria monocytogenes;* g= *Strepto coccus pyogenes;* h=Methicilline resistant *Staphylococcus aureus*

Non sensitive (-) : For total diameter smaller than 8 mm
Sensitive (+) : For total diameter between 9-14 mm
Very sensitive (++) : For total diameter between 15-19 mm
Extremely sensitive (+++): For total diameter larger than 20 mm

Table 3: Effect of dietary treatments on bacterial (CFU/ml)

	Control	T1	T2	T3	T4	T5	T6
Total count	242.5±4.83	101.75 ± 1.49	90 ± 2.52	99.5 ± 1.65	65 ± 4.67	82 ± 3.02	85 ± 1.84
Coliform	84.5±2.66	47.5 ± 2.39	74 ± 5.17	68.5 ± 2.87	52 ± 2.08	62± 1.93	60.5 ± 2.78
Salmonella	155.75±3.07	62.75 ± 7.04	66 ± 3.79	35.5 ± 3.17	28 ± 1.04	56.25 ± 2.52	44 ± 2.08
Staphylococcus	145±2.83	55± 4.14	61.25 ± 1.49	52.5 ± 2.39	23.5 ± 1.88	31.75 ± 1.49	40.5 ± 2.59
Mean*	157^{a} ±13.64	66.75^{b}±12.07	72.81^{b}±6.30	56.50^{b}±14.81	20.68^{b}±1.45	58.00^{b}±10.34	52.50^{b}±13.58

^{ab} values in a row not sharing common superscripts differ significantly * ($P<0.05$)

Table 4: Effect of dietary treatments on volatile fatty acid profile

	Control	T1	T2	T3	T4	T5	T6
Acetic acid (%)	58.60±0.88	61.7 ± 0.67	60.25 ± 2.86	64.43 ± 1.56	68.4± 1.16	63.25 ± 1.33	63.15± 1.50
Propionic acid (%)	24.20±1.03	25.63±1.57	20.08± 1.24	24.63 ± 2.65	20.08 ± 1.65	25.88 ± 1.34	24.28 ± 0.94
Iso butyric acid (%)	0.00±0	0 ± 0	1.6 ± 0.6	2.2 ± 0.92	0.3 ± 0.3	0.75 ± 0.43	1.75 0.32
Butyric acid (%)	18.00±0.36	12.55 ± 2.03	13.13 ± 2.83	8.8 ± 1.45	11.22± 0.3	10.28 ± 1.49	10.68 ± 1.41

It was documented that phytogenic feed additives have a strong antibacterial and to some extent antifungal properties. They inhibit the growth of *Escherichia coli*, *Proteus sp*, *Staphylococci*, *Streptococci* and *Salmonella* (Aruoma *et al.,* 1996; Benencia and Courreges, 2000; Garcia *et al.*, 2003) which otherwise compete with the host for nutrients.

Earlier reports also indicated antimicrobial effects of plants extracts (Newbold *et al.*, 2004). It was reported by Suryanarayana *et al.*, (2010) that herbal residues viz- *Emblica officinale, Zingiber officinale* and *Curcuma longa* were able to check the pathogenic load in the large intestines of finisher pigs.

3.2. Volatile fatty acid production

At the end of the fermentation, VFA profile was estimated (Fig. 2) to study the percentage of production of acetic acid, propionic acid, butyric acid and traces of iso butyric acid among treatments. In all the treatments, the range of production (%) of VFA (Table. 4) was 60-68, 20-25 and 10-13 for acetic, propionic and butyric-Iso butyric acids, respectively. However as compared to the control none of the treatments were found to be significant. In T_1 to T_6 acetic acid production dominated followed by propionic acid and butyric acid. T_4 has recorded higher acetic acid with a corresponding decrease in other fatty acids.

In the present findings, while studying the VFA profile, lactic acid did not find it's place. Bernalier *et al.*, (1999) reported that with the increase of duration of incubation, the lactic acid produced will get converted to acetic acid, propionic acid and butyric acid by some of the bacterial species like *propionibacterium spp*., *Clostridium spp* etc. These results are in agreement with Awati *et al.*, (2006) who reported that no lactic acid was found after 72 hours of fermentation. In the present study, since more organic matter and dry matter was fermented, VFA production was higher in T_4 as compared to other groups and vice-versa for T_5.

4. CONCLUSION

Residue of *Zingiber officinale* was able to inhibit the pathogenic load and it can be hypothesized that this residue will inhibit the pathogenic bacteria in gastro intestinal tract of pigs and improves productive indices.

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