In vitro fermentation characteristics of different carbohydrate sources in two dog breeds (German shepherd and Neapolitan mastiff)


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Introduction

A large population of micro-organisms, characterized by high density and wide diversity, inhabits the gut and forms a closely integrated ecological unit with the host. This complex mixed microbial culture could be considered as the most metabolically adaptable and rapidly renewable organ of the body (Simpson et al., 2002). The gut microbial community plays vital roles in physiological, nutritional and immunological function of the host (Mackie et al., 1997).

Even if the techniques developed by Hungate and refined by Bryant (1972) have allowed the publication of several information on the number, types and metabolic activities of bacteria that can be cultivated from the gut, only few papers have been published on the normal intestinal biota of canines (Clapper, 1970; Balish et al., 1977; Davis et al., 1977; Benno et al., 1992; Terada et al., 1992; Lewis...
et al., 1994; Willard, 1996; Raskin et al., 1997), unlike the wealth of information regarding livestock species. Most of the studies on canine faecal bacteria have been carried out with Beagles and less is known about other canine breeds. On the other hand, the phylogenetically based classification scheme was recognized only recently and most of knowledge of gut bacterial populations has been described using indirect microbiological techniques such as selective plate counts, selective enrichment, pure culture isolation and most probable numbers of estimates. The most comprehensive study regards the characterization of canine faecal bacterial population in three dog breeds [German shepherd (GS), English setter and miniature Schnauzer], using both cultivation-based and molecular techniques (Simpson et al., 2002).

The in vitro gas production technique (IVGPT) proposed by Theodorou et al. (1994) measures the kinetics of fermentation and could be used to assess the activity of microbial populations (Williams et al., 2001). The IVGPT involves measurements of accumulating gas during fermentation, to assess microbial activity of the population acting as a whole. At the end of the fermentation period, samples are taken for the measurements of the end products [volatile fatty acids (VFA), other gas (NH3)] and substrate utilization. The technique is carried out under strictly anaerobic conditions and is being used to estimate the activity of the microflora from many different sources, including rumen (Calabrò et al., 2004; Cutrignelli et al., 2007), different sections of gastrointestinal tract of pigs (Williams et al., 1997), rabbits (Bovera et al., 2008a,b), caecum of poultry (Williams et al., 1995; Bovera et al., 2007) and faeces of dog (Cuterignelli, 2006). By using different substrates, it becomes possible to look at shifts in microbial populations which are associated with the fermentation of a particular feedstuff. It could be questioned whether the use of faeces as inoculum for in vitro studies of in vivo intestinal events is truly representative for the fermentation earlier in the gastrointestinal tract (caecum or colon). Drasar (1988) described faeces as the spent culture medium of the large gut fermenter; it has been studied because it is readily available and provides a source material for the major groups of intestinal bacteria; it can also be sampled from the same host at different times. In detailed microbial count studies, Moore et al. (1978) concluded that the bacterial flora composition of faeces resembled that of large intestine and that freshly passed faeces collected under strictly anaerobic conditions could be considered as representative of large-intestinal flora. In terms of VFA and cumulative gas production, some differences were found between incola from caecum and faeces (Williams et al., 1997), but it was concluded that faeces did nevertheless give a reasonable estimate of activity in the upper put of caecum-colon.

The aim of this study was to study the fermentation characteristics of different carbohydrate sources by IVGPT using faecal incola from GS and Neapolitan mastiff (NM) dogs.

Material and methods

For the trial, two GS (3 years old) and two NM (3 years old) were utilized. The mean body weights of the animals were 32.5 kg and 59.8 kg for GS and NM respectively. The dogs raised in the same kennel were fed (140 kcal of ME/kg0.75) a commercial dry food (crude protein 25.8% as fed; total dietary fibre 6.6% as fed). After 20 days of adaptation, faecal samples were collected per rectum and immediately transported, under anaerobic condition, to the laboratory.

Faecal samples were diluted (1:10) with NaCl solution, homogenized, filtered and incubated at 39 °C under anaerobic condition in 120 ml serum bottles (Bauer et al., 2001). Gas production of fermenting cultures was recorded 17 times (at 2–4 h intervals) using a manual pressure transducer. The fermentation was stopped after 48 h, and the fermenting liquor was analysed for pH (Alessandrini Instrument glass electrode; Jenway, Dunmow, UK; model 3030) and VFA. For VFA determination, the sample was centrifuged twice at 12 000 × g for 10 min at 4 °C and 1 ml of supernatant was taken and mixed with 1 ml of oxalic acid (0.06 mol). The VFA were measured by gas chromatography (ThermoQuest Italia SpA, Rodano, Milan, Italy; model. 8000top, fused silica capillary column 30 m × 0.25 mm × 0.25 µm film thickness) comparing samples peaks area of each VFA with the corresponding of an external standard composed by acetate, propionate, butyrate, isobutyrate, valerate and isovalerate (Calabrò et al., 2006).

Organic matter disappearance (OMD) was determined by filtering under vacuum on pre-weighted glass crucibles (Scott Duran, porosity #2) the fermentation residues, which was dried at 103 °C and burning the residual at 550 °C.

Gas volumes recorded during the fermentation were related to the quantity of incubated OM (organic matter cumulative volume, OMVC). The gas profiles were fitted to the model described by Groot et al. (1996) as follows:
Carbohydrate fermentation in dog

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G = A/[1 + (B/t)C]

where G: total gas produced (ml/g), A: asymptotic gas production (ml/g), B: time at which one-half of the asymptote has been reached (h), C: switching characteristic of the curve and t: time (h).

Maximum fermentation rate \( R_{\text{max}} \) and time at which it occurs \( T_{\text{max}} \) were also calculated according to the following formulas (Bauer et al., 2001):

\[
R_{\text{max}} = (A \cdot C^B \cdot B \cdot [(1 + C^B)(T_{\text{max}}^B)]^2
\]

\[
T_{\text{max}} = C \cdot [(B - 1)/(B + 1)]^{1/2}\]

Nine substrates representative of carbohydrates with different fermentation characteristics were used: rice (R); corn (C); spelt (S); potato (P); sugar beet pulp (BP); wheat bran (WB); fructo-oligosaccharides (FOS); inulin (I) and pure cellulose (PC). Before the trial all substrates were analysed for the chemical composition (AOAC 2006); starch was determined using a polarimeter (Polax-L, model PX-L, ATAGO Co, LTD, Tokyo, Japan) (Martillotti et al., 1987).

The fermentation characteristics and the fitted parameters were subjected to analysis of variance (GLM procedure of SAS 2000) to detect the breed (MN and GS) and substrate (R, C, S, P, BP, WB, FOS, I and PC) effects; in the model the breed \( \times \) substrate interaction was included. Pearson correlation coefficients between chemical composition and in vitro parameters and among the fermentation parameters were studied using the CORR procedure of SAS (2000).

**Results**

In Table 1, the chemical composition of the substrates is shown. Generally, chemical composition of the nine substrates agrees with the data reported in bibliography (INRA, Institut National de la Recherche Agronomique, 1987; Martillotti et al., 1989; Piccioni, 1989).

In Table 2, the fermentation characteristics are shown. As expected, IVGPT parameters were significantly influenced by substrates chemical composition: OMD was significantly \((p < 0.01)\) higher for FOS and inulin (99.14 and 94.89% respectively), while cellulose gave the lowest value (2.50%). The substrates richer in starch (C, R, P, S) showed the highest values of OMCV. Concerning the mathematical model parameters rice, corn, spelt and potato showed significantly higher values of \( R_{\text{max}} \) than inulin and FOS, while the latest were characterized by significantly \((p < 0.01)\) higher values of \( R_{\text{max}} \).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dry matter</th>
<th>Crude protein</th>
<th>Ether extract</th>
<th>TDF</th>
<th>Ash</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>87.53</td>
<td>6.49</td>
<td>0.66</td>
<td>6.42</td>
<td>0.53</td>
<td>73.43</td>
</tr>
<tr>
<td>C</td>
<td>88.39</td>
<td>7.76</td>
<td>1.03</td>
<td>8.04</td>
<td>1.41</td>
<td>70.15</td>
</tr>
<tr>
<td>P</td>
<td>87.81</td>
<td>8.45</td>
<td>0.21</td>
<td>13.2</td>
<td>4.78</td>
<td>61.16</td>
</tr>
<tr>
<td>S</td>
<td>88.20</td>
<td>13.7</td>
<td>1.66</td>
<td>12.2</td>
<td>1.50</td>
<td>59.24</td>
</tr>
<tr>
<td>PC</td>
<td>95.63</td>
<td>–</td>
<td>0.04</td>
<td>94.4</td>
<td>0.20</td>
<td>–</td>
</tr>
<tr>
<td>BP</td>
<td>90.48</td>
<td>7.51</td>
<td>0.55</td>
<td>77.8</td>
<td>3.43</td>
<td>1.170</td>
</tr>
<tr>
<td>WB</td>
<td>88.28</td>
<td>15.2</td>
<td>1.98</td>
<td>50.9</td>
<td>4.34</td>
<td>15.82</td>
</tr>
<tr>
<td>I</td>
<td>97.73</td>
<td>–</td>
<td>0.04</td>
<td>97.5</td>
<td>0.15</td>
<td>–</td>
</tr>
<tr>
<td>FOS</td>
<td>95.90</td>
<td>0.03</td>
<td>0.06</td>
<td>95.7</td>
<td>0.11</td>
<td>–</td>
</tr>
</tbody>
</table>

Concerning the comparison between breeds, the inoculum from NM gave significantly higher \((p < 0.01)\) values of OMD (74.48 vs. 72.17%), B (16.15 vs. 12.56 h), C (2.45 vs. 2.15) and \( T_{\text{max}} \) (10.50 vs. 7.82 h) and lower of OMCV (138.9 vs. 157.6 ml/g) than GS.

Interesting correlation were found among the fermentation parameters and some chemical characteristics (Table 3). In particular, the correlation coefficients between TDF and several fermentation parameters gave high \( r \) values and were significant for OMCV, A (\( p < 0.01 \)) and \( T_{\text{max}} \) (\( p < 0.05 \)), while starch content was significantly (\( p < 0.05 \)) correlated with OMCV, A and \( T_{\text{max}} \). Protein and ether extract contents resulted significantly correlated with any fermentation parameter.

Values of pH measured at the end of the fermentation and VFA (mmol) concentration, and their correlation with chemical composition values were shown in Tables 4 and 5 respectively.

The pH determined after 48 h of incubation resulted particularly low for corn and inulin (4.52 and 4.72 respectively) and quite high for pure cellulose (7.06) and results significantly (\( p < 0.01 \)) correlated with propionate \(( r : -0.676 \)) and isovalerate \(( r : 0.791 \)) concentrations.

Volatile fatty acids concentrations were significantly correlated with chemical composition parameters, while carbohydrate fractions influenced (\( p < 0.01 \) and \( p < 0.05 \)) acetate and propionate productions; crude protein and ether extract were related to isobutyrate, butyrate and valerate production. Total VFA production resulted significantly (\( p < 0.01 \)) higher for R, S and P (58.46, 58.00, 57.64 and 49.58 mmol respectively) than the other substrates, principally because of the higher concentrations of acetate and propionate. As expected, pure
cellulose gave the lowest total VFA concentration (15.12 m); however, this substrate was characterized by higher valeric and isovaleric acids concentrations. Because of the lack of available nutrients, the microbial growth would be reduced and even microbial lysis could occur. It seems interesting to evidence that total VFA values for beet pulp and wheat bran resulted higher than inulin and FOS, as a result of the high fermentability of BP fibre and the relatively high starch content of WB (15.82% as fed). The low VFA production of FOS and I were probably caused by the nitrogen lack of these substrates.

At the end of the fermentation, the inoculum from NM produced more VFA than GS inoculum (47.51 vs. 38.78 mmol; p < 0.01) and the differences have to be ascribed exclusively to the propionate which in NM was approximately twice as in GS. On the other hand, the concentrations of isobutyric, isovaleric and valeric acids were significantly (p < 0.01) higher for GS than NM. It seems interesting to underline that the inocula showed different trends from those of corn and spelt, probably because of the differences in starch fermentability. Regarding BP, PC and WB, the gas profiles of the two breeds were similar, while among feedstuffs the curve shapes were different. As expected, FOS and inulin gave similar

Table 2 In vitro fermentation parameters of the substrates

<table>
<thead>
<tr>
<th>Substrate effect (mSD)</th>
<th>OMD (%)</th>
<th>OMVC (ml/g)</th>
<th>A (ml/g)</th>
<th>B (h)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>86.46± 2.65</td>
<td>206.5± 30.04</td>
<td>208.14± 28.22</td>
<td>14.64± 5.41</td>
<td>9.11&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>4.38</td>
</tr>
<tr>
<td>P</td>
<td>88.29± 1.88</td>
<td>179.9± 8.70</td>
<td>209.87± 12.24</td>
<td>21.11&lt;sup&gt;AA&lt;/sup&gt;</td>
<td>3.56</td>
<td>15.14&lt;sup&gt;AA&lt;/sup&gt;</td>
</tr>
<tr>
<td>S</td>
<td>88.15± 2.39</td>
<td>206.9± 10.45</td>
<td>212.12± 10.87</td>
<td>17.09&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.16</td>
<td>13.7&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC</td>
<td>2.5± 0.13</td>
<td>3.6± 0.17</td>
<td>3.53± 0.22</td>
<td>8.06&lt;sup&gt;D&lt;/sup&gt;</td>
<td>2.70</td>
<td>3.91&lt;sup&gt;D&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP</td>
<td>46.81± 3.25</td>
<td>129.1± 19.35</td>
<td>140.50± 12.09</td>
<td>23.72± 4.59</td>
<td>11.77&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.58</td>
</tr>
<tr>
<td>WB</td>
<td>63.56± 1.96</td>
<td>146.6± 20.3</td>
<td>155.63± 12.49</td>
<td>6.31&lt;sup&gt;D&lt;/sup&gt;</td>
<td>1.17</td>
<td>2.49&lt;sup&gt;D&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>94.89± 2.76</td>
<td>158.3± 28.99</td>
<td>160.12± 30.16</td>
<td>8.06&lt;sup&gt;D&lt;/sup&gt;</td>
<td>0.98</td>
<td>5.67&lt;sup&gt;CD&lt;/sup&gt;</td>
</tr>
<tr>
<td>FOS</td>
<td>99.14± 0.78</td>
<td>134.0± 33.32</td>
<td>140.71± 33.42</td>
<td>7.99&lt;sup&gt;D&lt;/sup&gt;</td>
<td>1.41</td>
<td>5.35&lt;sup&gt;CD&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Breed effect

<table>
<thead>
<tr>
<th>Breeds</th>
<th>OMD (%)</th>
<th>OMVC (ml/g)</th>
<th>A (ml/g)</th>
<th>B (h)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM</td>
<td>74.48± 28.07</td>
<td>138.9± 60.04</td>
<td>154.5± 66.00</td>
<td>16.15± 8.00</td>
<td>10.50&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.16</td>
</tr>
<tr>
<td>GS</td>
<td>72.17± 29.20</td>
<td>157.6± 65.22</td>
<td>163.3± 66.27</td>
<td>12.56± 5.96</td>
<td>7.82&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>4.25</td>
</tr>
</tbody>
</table>

Int. 0.3096 0.1751 0.0272 <.0001 0.0026 0.0380

Values are given as mean ± SD. R, rice; C, corn; P, potato; S, spelt; PC, pure cellulose; BP, beet pulp; WB, wheat bran; I, inulin; FOS, fructo-oligosaccharides; OMD, organic matter digestibility; OMVC, organic matter cumulative volume; A, asymptotic gas production; B, time at which one-half of the asymptote has been reached; C, switching characteristic of the curve; R<sub>max</sub>, maximum fermentation rate; T<sub>max</sub>, time at which R<sub>max</sub> occurs; GS, German shepherd; NM, Neapolitan mastiff; Int., interaction breed x substrate.

A–Ep < 0.01.

Table 3 Correlation among chemical characteristics and fermentation parameters

<table>
<thead>
<tr>
<th>OMD</th>
<th>OMVC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>T&lt;sub&gt;max&lt;/sub&gt;</th>
<th>R&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>TDF</td>
<td>0.643</td>
<td>NS</td>
<td>0.904</td>
<td>&lt;0.01</td>
<td>0.935</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Starch</td>
<td>0.406</td>
<td>NS</td>
<td>0.687</td>
<td>&lt;0.05</td>
<td>0.730</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CP</td>
<td>0.135</td>
<td>NS</td>
<td>0.520</td>
<td>NS</td>
<td>0.537</td>
<td>N</td>
</tr>
<tr>
<td>EE</td>
<td>0.099</td>
<td>NS</td>
<td>0.428</td>
<td>NS</td>
<td>0.398</td>
<td>NS</td>
</tr>
</tbody>
</table>

TDF, total dietary fibre; CP, crude protein; EE, ether extract; OMD, organic matter digestibility; OMVC, organic matter cumulative volume; A, asymptotic gas production; B, time at which one-half of the asymptote has been reached; C, switching characteristic of the curve; R<sub>max</sub>, maximum fermentation rate; T<sub>max</sub>, time at which R<sub>max</sub> occurs; r, correlation coefficient; NS, not significant.

Correlation among chemical characteristics and fermentation parameters

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>OMD (%)</th>
<th>OMVC (ml/g)</th>
<th>A (ml/g)</th>
<th>B (h)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>R&lt;sub&gt;max&lt;/sub&gt; (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>EE</td>
<td>0.997</td>
<td>&lt;0.001</td>
<td>0.645</td>
<td>NS</td>
<td>0.571</td>
<td>NS</td>
</tr>
<tr>
<td>Starch</td>
<td>0.406</td>
<td>NS</td>
<td>0.687</td>
<td>&lt;0.05</td>
<td>0.730</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

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trend in terms of gas production and fermentation rate.

In Table 6, the correlations between fermentation parameters and end-products were reported. OMD was significantly (p < 0.01) correlated with OMCV and the ratio among acetate plus butyrate (the VFA more involved in gas production), and propionate (the VFA which give less gas production). OMCV was significantly (p < 0.01) related with all considered parameters.

Discussion and conclusion

The gas production and the OM digestibility registered within 48 h with GS inoculum for pure cellulose, FOS, inulin and wheat bran were in accordance with that of a previous study (Cutrignelli, 2006) carried out using faecal inoculum from the same breed. The high correlation among carbohydrate composition (TDF and starch) and in vitro parameters confirm the suitability of IVGPT to study the fermentation characteristics of feed for dogs.

The inocula showed low cellulolytic activity, in both case PC was digested only in minimal portion, according to Sunvold et al. (1995), while the percentages of OMD for FOS, I and for substrates rich in starch (P, S, C and R) were high, demonstrating high microbial activity on non-structural carbohydrates.

However, the differences registered between breeds in fermentation parameters, end-products and fermentation kinetics suggest different pathways of carbohydrate fermentation, in particular for substrates rich in starch and confirm the observation of Simpson et al. (2002), who found significant differences among breeds on selected aerobic and anaerobic bacterial counts.

Faeces from NM degraded the OM more intensively, but gas produced along the fermentations (OMCV, A) was lower than from GS faeces. However, the higher OM degradation was not imputable for pure cellulose inoculum from the NM breed. The high correlation in aerobic bacterial counts.
Fig. 1 In vitro gas production and fermentation rate over time of the substrates in German shepherd (GS) and Neapolitan mastiff (NM) dogs.
to protein degradation, but to the different utilization of non-structural carbohydrates. It is worth pointing out that isobutyric, isovaleric and valeric acids came from the bacterial metabolism of valine, leucine and proline respectively. Therefore, on the same fermented substrates, faeces from GS degraded the proteins more intensively than NM. The non-significant interactions between the effects suggest that the inocula have the same behaviour, irrespective of the differences among substrates. Indeed faecal inoculum from NM seems more able to utilize some substrates producing high proportion of propionate and consequently low gas. It is known that the production of propionate is tied to a lower production of gas (CO₂). Actually, acetate production is correlated with one molecule of CO₂ and one of H₂, while that one of propionate is only correlated with 1 molecule of H₂O. In this case, the interaction was statistically significant due to the particular behaviour of cellulose which showed an inverse trend compared with the other substrates, because it was the only one purified. As a consequence, the two breeds seem very different in terms of fermentation capability, probably due to a different microbial population or different microbial activity.

These preliminary results could be considered to be the first step to study the gastrointestinal microbiota activity in dogs using the gas production technique.

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