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Anxiety and aggression associated with the fermentation of carbohydrates in the hindgut of rats

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Abstract

Lactic acid accumulation in the caecum and colon resulting from the fermentation of carbohydrates can lead to deleterious effects in ruminant and monogastric animals, including humans. In the present study, we examined the behavioural effects of two types of commonly consumed foods: soluble and fermentable carbohydrates (FCs). Thirty-six male Wistar rats were fed either a commercial rat and mouse chow, a soluble carbohydrate (SC)-based diet or an FC-based diet. Social interaction, anxiety, aggression and locomotor activity were examined by employing a social interaction test and a light/dark emergence test, while physical parameters of hindgut fermentation were examined after sacrifice, either 3 or 21 h after feeding. Results showed that anxiety (spending less time in the light compartment during the light/dark emergence test) and aggression (increased fighting during the social interaction test) were increased following raised concentrations of fermentation end products, such as lactic acid and volatile fatty acids (VFAs) in the caecum of rats. These associations occurred regardless of dopamine and 5-HT concentrations in the prefrontal cortex (PFC) and provide evidence supporting a general effect of FCs on behaviour. Possible mechanisms of action along with similarities between a rat and human model of acidosis are discussed.

Keywords: Aggression; Anxiety; Caecum; Hindgut acidosis; Lactate; Light/dark emergence test; Rats; Serotonin; Social interaction test

1. Introduction

Investigation into the effects of carbohydrate intake on behaviour and mood has centred on the level of serotonin (5-HT) and its precursor, tryptophan, in the brain 2–4 h after carbohydrate consumption [1,2]. An increased concentration of 5-HT in the brain has been associated with feelings of calmness, sleepiness and well-being in some people [3–5]. However, previous researchers have not considered whether the site of digestion may influence behavioural effects or mediate brain chemistry alterations. The majority of researchers typically label all carbohydrates as purely 'carbohydrates' and do not specify whether they are, in fact, examining soluble or fermentable carbohydrates (FCs). These two types of carbohydrates differ in the site of digestion, with soluble carbohydrates (SCs) being digested in the stomach and small intestine and FCs in the caecum and colon or hindgut [6].

Fermentative acidosis resulting from rapid fermentation of carbohydrate and the production of large quantities of organic acids such as volatile fatty acids (VFAs) and lactic acid occurs in the hindgut of many animals including ruminants [7–9] and monogastrics including pigs [10], horses [11,12], rats [13,14] and humans (for a review, see Ref. [15]). Animal research has consistently demonstrated that lactic acidosis can detrimentally affect an animal's physical health and is associated with laminitis [16,17], liver abscess [18] and respiratory disease [19] in cattle, laminitis in horses [11,20], swine dysentery in pigs [21], inflammation and immune function in sheep [22] and gut inflammation in poultry [23]. Additionally, acid accumulation and acidosis affect many metabolic systems in humans and is involved with bone resorption [24,25].

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Acute D-lactic acidosis, resulting from fermentation in the hindgut of patients with shortened small intestines (short bowel syndrome) or with jejunoileal bypass for morbid obesity, occurs due to the large amount of undigested carbohydrate entering the caecum and large intestine that would normally be absorbed in the foregut (for a review, see Ref. [15]). Although this is an acute and somewhat unnatural condition in humans, this model can be used to study the effects of acid accumulation on behaviour. D-Lactic acidosis produces many abnormal physical and behavioural effects including ataxia, nystagmus, slurred speech, blurred vision, aggressive behaviour, nausea and vomiting (for a review, see Refs. [15,26]). Three undiagnosed cases of D-lactic acidosis in patients with short bowel syndrome were also associated with symptoms of dementia, which improved when corrective action for the acidosis was taken [27].

Panic attacks in patients prone to panic attack disorder can be initiated by intravenous infusion of lactic acid [28]. Recently, researchers have indicated that only patients prone to panic attack disorder are affected by lactic acid infusion [29,30]. However, work by Pitts and McClure [28] and Balon et al. [31] indicate that a small percentage of control patients also exhibited signs of panic and many control patients described symptoms associated with heightened anxiety including increased heart rate and blood pressure and feelings of tightness in the chest [32]. The mechanism of action leading from intravenous infusion of lactic acid to the observed cognitive and behavioural responses is not known. Likewise, the effects of lactic acid arising from the fermentation of carbohydrate in the hindgut on anxiety or panic attack occurrence are not known. It is well accepted that acid accumulation in the hindgut can result in systemic acid base disturbance and metabolic acidosis (e.g., see Ref. [33]).

The human hindgut is physiologically similar to animals with respects to fermentation, in that VFA and increased lactic acid can be produced from an overload of complex carbohydrates [34,35]. No previous studies have specifically investigated the negative effects of fermentative acidosis on human cognitive performance and behaviours, such as anxiety and aggression.

The purpose of the present study is to examine the link between acid accumulation in the hindgut of rats following the fermentation of commonly consumed carbohydrate sources on behavioural parameters indicating anxiety and aggression. It is predicted that acid accumulation in the hindgut of rats can produce behavioural problems, independent of changes in brain 5-HT concentrations that may occur 2–4 h after carbohydrate intake.

2. Materials and methods

2.1. Subjects

Thirty-six male albino Wistar rats, 6 weeks old upon arrival to the colony, were used. All animals were housed in groups of six according to diet in opaque polypropylene cages [64×41×25 cm high] with stainless steel lids during a 2-week acclimation period and throughout the 3-day testing phase. Cages were lined with dust-free wood chips and were housed in a climate-controlled room maintained on a 12:12-h reverse light–dark cycle (lights off at 0800 h). Tap water was available ad libitum throughout the experiment. Rats were fed in their group cages every second day and on each alternate day were placed in single cages for their meal from 1100 to 1400 h. After feeding, animals were returned to their group cages. All experimental procedures were approved by the University of New England Animal Ethics Committee and were conducted in compliance with the Animal Research Act 1985 (as amended).

2.2. Dietary treatments

Rats were fed three different diets during the experimental period (n=12 per dietary treatment). The aim of the choice of diets was to provide a range of carbohydrate sources that were expected to be digested in different sections of the gastrointestinal tract. A wheat-based diet (M. Evans, Applied Nutrition, QLD) was used as the SC diet and was likely to be digested predominantly in the stomach and small intestine. The FC diet was formulated specifically for the experiment and was based on the main ingredient of cooked and cooled rice (E. Clayton, TLC Research and M. Evans, Applied Nutrition, QLD). This diet was expected to be relatively higher in retrograded starch after cooking and cooling and was likely to be fermented in the caecum and colon or hindgut. The control diet was a commercially available rat pellet (“Rat and Mouse Chow” Ridley Agriproducts, NSW, Australia) (Chow) routinely used in the laboratory. The likely extent of fermentation of the Chow diet in the hindgut could not be accurately predicted; however, it was expected to be different than the other two diets. The three diets were formulated using the International FORMAT feed formulation software package and conformed to NRC guidelines for rodent diets. Table 1 presents the specifications of the three test diets.

During the first week of habituation, rats were randomly assigned to three groups of 12, matched for body weights. All rats were maintained on the Chow diet from the time of weaning. In the second week of laboratory habituation and 8 days prior to conducting the behavioural tests, rats in the SC and FC groups were introduced to their test diets in order to overcome flavour neophobia during the experimental phase. Rats in the Chow diet group remained on chow throughout the habituation and experimental periods. It was expected that the SC component of the diets would be digested approximately 3 h after feeding [1] and the FC components would be digested approximately 21 h after feeding, given the gut transit time of the rat from mouth to caecum/colon of approximately 18–24 h [13]. Therefore, during the experimental period, rats were tested in behavioural tests or euthanised for collection of gut contents for

Table 1
Components, specifications and laboratory analysis[a] of protein, fat and fibre for the diets used in the study

Ingredients	Units	Chow	SC	FC
Wheat	%	[b]	61.00	–
Rice	%	–	–	65.00
Millrun	%	[b]	15.00	–
Lupins	%	–	3.45	10.00
Cottonseed meal	%	[b]	3.00	–
Soybean meal	%	[b]	4.00	14.00
Full fat soybean meal	%	[b]	–	–
Sunflower meal	%	[b]	–	–
Blood meal	%	–	2.15	–
Meat meal	%	[b]	5.00	6.75
Vegetable oil	%	–	1.60	–
Molasses—cane	%	[b]	–	–
Limestone fine	%	[b]	0.80	0.25
Salt	%	[b]	1.10	1.18
Causmag	%	–	1.00	–
Choline chloride	%	–	0.20	0.17
DL methionine	%	–	0.40	0.44
Lysine HCl	%	[b]	0.10	0.02
Sodium bentonite	%	[b]	1.00	2.00
Premix	%	[b]	0.20	0.20
Analysis				
Acid detergent fibre	%	10.2	4.30	2.30
Neutral detergent fibre	%	29.4	14.8	15.1
Protein	%	22.4	19.1	18.2
Fat	%	3.80	1.70	1.4
Crude fibre	%	6.70	4.40	2.3
DE	MJ/kg DM	16.6	17.1	16.9
Specifications				
Ca	%	1.13	1.00	0.98
P	%	0.86	0.53	0.66
Na	%	0.25	0.50	0.47
K	%	1.03	0.77	0.57
Cl	%	0.43	0.83	0.80
Lysine	%	1.20	0.92	0.93
Methionine	%	0.33	0.71	0.66
Cysteine	%	0.47	0.23	0.34
Threonine	%	0.78	0.63	0.63
Leucine	%	1.53	1.36	1.30
Isoleucine	%	0.88	0.78	0.63
Tryptophan	%	0.26	0.21	0.20
Linoleic	%	1.40	0.73	2.08
Choline	%	1414	2022	1982
Vitamin A	IU/g	11.14	7.09	2.57
Vitamin D3	IU/g	2.00	2.40	4.00
Vitamin E	mg/kg	66.17	21.13	15.52
Vitamin K	mg/kg	0.50	0.60	0.50
Vitamin B1 (thiamine)	mg/kg	19.18	2.63	5.36
Vitamin B2 (riboflavin)	mg/kg	12.83	7.13	1.88
Vitamin B3 (niacin)	mg/kg	114.91	40.98	55.26
Vitamin B5 (pantothenic acid)	mg/kg	27.30	20.44	12.78
Vitamin B6 (Pyridoxine)	mg/kg	11.28	3.28	3.49
Vitamin B12	mg/kg	0.04	0.020	0.020
Biotin	mg/kg	0.39	0.09	0.10
Folic	mg/kg	2.97	0.74	0.60
Fe	mg/kg	279.82	100.46	160.63
Zn	mg/kg	260.16	17.01	23.61
Mn	mg/kg	134.20	17.61	50.10
Cu	mg/kg	34.27	5.28	8.29
Se	mg/kg	0.64	0.10	0.23

assessment of fermentation parameters either 3 or 21 h after presentation of the feed. Rats were placed in the social interaction test and then in the light/dark test 3 h after feeding on Day 0 (Fig. 1) and were then placed in the two tests in the same order 21 h after the feed was presented on Day 1 (Fig. 1). Half of the rats in each dietary group were stratified and randomly tested at 3 h then 21 h or vice versa.

2.3. Apparatus

2.3.1. The social interaction test

The testing arena consisted of a clear glass box (62×30×36 cm high) with a glass floor. Existing room lights were used for illumination yielding approximately 350 lx in the test area. All sessions were recorded using a black and white CCD camera, which was connected to a VCR.

2.3.2. The light/dark emergence test

The apparatus consisted of a box with two distinct chambers; an aluminium dark box (26×9×10 cm high) and a brightly lit Perspex box (29×29×39 cm high) connected by a small open doorway (7.5×5 cm high). A 60-W light was placed directly in front of the open lit compartment at a distance of 100 cm and above the level of the testing arena. The dark compartment had two infrared beams located 10 cm apart, which were monitored by a computer to determine the location of the rat. A passive infrared sensor was mounted to the ceiling of the light compartment and was also monitored by a computer. Custom-written software was used to record the time spent in the light compartment as well as the time spent in motion (an index of locomotion) in the light compartment.

2.4. Procedure

2.4.1. Social interaction

Each test rat was randomly allocated with an unfamiliar age- and weight-matched nontest male Wistar rat. The day prior to testing, each rat (both the test rat and the unfamiliar conspecific) was placed alone in the testing arena for a 10-min habituation session. This session was conducted to enhance the novelty of the unfamiliar rat relative to the testing arena during subsequent experimental sessions [36]. The following day, the pair of rats was placed in the centre of the test arena and their behaviour was recorded onto videotape for 10 min for subsequent scoring. After each trial, the testing arena was cleaned with a household cleaning solution (Home Brand Glass Cleaner, Woolworth's

Notes to Table 1:
SC=Soluble carbohydrate diet based on wheat; FC=fermentable carbohydrate diet based on cooked and cooled rice; DE=digestible energy calculated according to the formula used by Zijlstra et al. [76].
[a] Source: Weston Animal Nutrition Analytical Laboratory.
[b] Dietary ingredient included but inclusion percentage is unavailable.

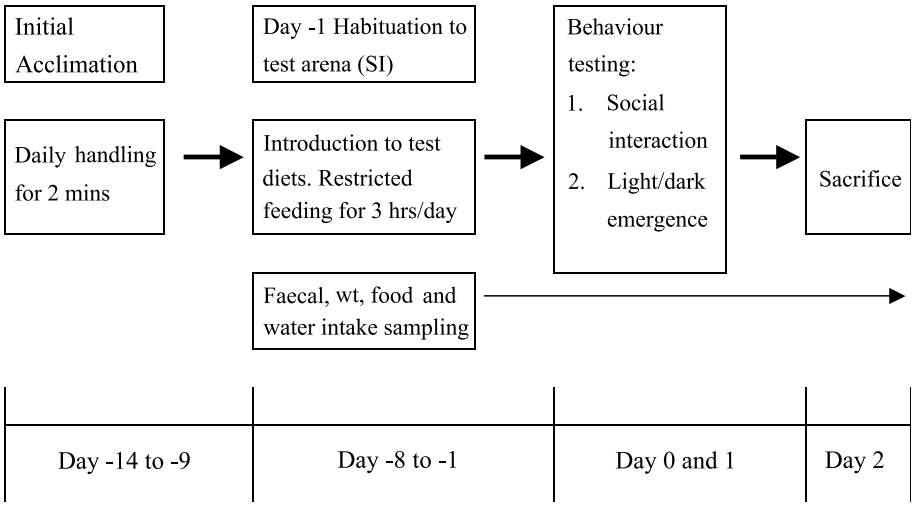

Fig. 1. The sequential order of subject manipulation, behaviour testing and sacrificing.

Grocery Wholesalers, Yennora, NSW). Each rat was tested twice, 3 and 21 h after feeding the test diets, with a new partner each time.

Data were scored from the videotape footage using ODLog for Windows [37]. The frequency and duration of each type of social behaviour performed by the test rat was scored. Social interaction was coded into investigative behaviour (sniffing, following, grooming, crawling under/over), fighting behaviour (mounting/jumping on, biting/pulling, wrestling/boxing, pinning), rearing (rat on hind paws only, either in the centre of the arena or leaning against the wall) and total social interaction score (total of investigative and fighting behaviour). A further analysis distinguished fighting behaviour into two categories; firstly, playful fighting (mounting/jumping on, wrestling/boxing) and secondly, more serious fighting (pinning, biting/pulling).

The cumulative frequency and duration of time that the rat engaged in locomotor activity during the 10-min social interaction test were also scored. In an attempt to provide a similar measure of locomotor activity to that used in the light/dark emergence test, movement was scored when the rat moved both horizontally (ambulation) and vertically (rearing).

2.4.2. Light/dark emergence test

Rats were placed individually in the centre of the light compartment (facing away from the partition opening) both 3 and 21 h after receiving the test diets. The rats were allowed to explore the apparatus undisturbed for 15 min. After each testing session, the apparatus was cleaned thoroughly to mask any odour trails left by the rat in the previous trial, using the household glass cleaning solution described above.

Sensors were used to record the time in the light compartment (light time) and the number of transitions between compartments by breaking one of two beams: the outer beam (leaving the light box into the passageway) and inner beam (entering the inner dark box from the passageway). Locomotor activity in the brightly lit compartment was also automatically detected. Data were automatically stored in a text file for subsequent analysis. The light time sensor stopped registering when the rat broke the outer beam and there was no activity observed in the light compartment.

2.5. Samples and collections

2.5.1. Body weight, feed and water intake

Rat weights were monitored every second day for 2 weeks during the acclimation phase as well as throughout the experimental testing period. Rats were placed in single cages between 1100 and 1400 h every second day for individual measurement of test diet and water consumption. The feed was weighed before presentation to the rat and remaining feed and spillage were weighed after each feeding period. Water containers were also weighed before and after feeding to calculate water intake. Daily individual intake was calculated from the feed offered minus feed refused. On each alternate day when the rats were group fed, food intake was calculated by dividing total feed intake by the number of rats in each cage [6].

2.5.2. Faecal collection and pH

A faecal sample was collected fresh from the cage on each day when the rats were individually fed. Additionally, where possible, a faecal sample was collected from the floor of the experimental chamber after the rats had completed their behavioural tests. The samples were placed in 5-ml plastic specimen containers and frozen at −18 °C until analyzed. For the analysis, once thawed, each faecal sample was mixed with doubled distilled water (ddH_2O) 2:1 (w/w), a glass bead was added and the contents were mixed with a vortex mixer. The pH of the faecal samples and gut contents

was measured using a combination glass calomel pH probe (Selby Biolab).

2.5.3. Euthanasia and collection of brain and gut samples

On the day after behavioural testing, six rats from each diet group were euthanised 3 h after receiving their test diets. The remaining six rats from each diet group were euthanised 21 h after receiving their test diets. Rats were euthanised by brief exposure to CO_2 gas. The brains were rapidly removed, dissected into left and right lobes and placed in 5-ml plastic specimen containers, which were sealed before being submerged in liquid nitrogen. Brains were stored at −80 °C until analyses were performed. The ileum, caecum, proximal and distal colon (including rectum) were removed and weighed and observed for signs of gross lesions and abnormalities. The entire colon from the caecal junction to the anus was removed and laid out straight. The proximal colon was taken as the first physical half of the colon, while the distal colon was taken as the second half of the total colon segment. Contents from each gut section were removed and placed in 5-ml plastic specimen containers. Proximal and distal colon samples were mixed 1:2 (w/w) with distilled water. Samples were mixed thoroughly to provide a consistent sample, and pH was measured immediately on all samples prior to the samples being frozen at −18 °C for later analysis.

2.6. Laboratory analyses

2.6.1. Volatile fatty acids

Concentrations of VFAs were measured in each sample of digesta from the ileum, caecum, proximal colon and distal colon using a methodology for capillary gas chromatography (GC) provided by M. Davies, Agriculture Victoria, Ellinbank Laboratory, Victoria. In brief, contents of ileum, caecum, proximal and distal colon were thawed and mixed with a 10% formic acid/10% orthophosphoric acid plus 4-methyl valeric acid at 184 ppm (as internal standard) solution 2:1 (w/w). A glass bead was added and the contents mixed with a vortex mixer. Once mixed, samples were centrifuged at 20 000×*g* for 20 min in a Biofuge Stratos centrifuge (Kendo Laboratory Products, Hanau, Germany). A 500-μl aliquot was added to a GC vial. VFA concentration was determined by capillary GC using a Hewlett Packard 6890 Series Gas Chromatograph with H-P 7683 Injector/Auto-sampler (H-P Company, 2850 Centreville Rd, Wilmington DE, USA). The method employed a SGE BP21 analytical column, fitted with a retention gap kit and an SGE RGK2 guard column of 1 m. An inlet volume of 1 μl at an inlet temperature of 155 °C and pressure of 8.2 kPa was used. The total flow was 48.7 ml min^{-1} and split ratio of 5:1. The carrier gas was ultrapure H. Data were initially analysed as concentration (ppm) and converted to millimole for further analysis.

2.6.2. Lactic acid

All digesta samples were handled as for the VFA analysis. The concentrations of D- and L-lactic acid were measured in each sample with a Cobas-Bio (Roche Diagnostic Systems) using specific D- and L-lactic acid dehydrogenases and the ultra violet (UV) detection method (Cat. No. 1 112 821, r-Biopharm, Darmstadt, Germany).

2.6.3. Neurochemistry

The prefrontal cortex (PFC) was dissected from the brain while still frozen. Dissection took place on a glass plate on frozen carbon dioxide. Samples were individually placed in plastic microcentrifuge tubes and stored at −80 °C until assayed.

Tissue samples were homogenised with a 500-μl ice-cold solution of 0.2 M perchloric acid containing 0.1% cysteine and 200 nmol/l of internal standard 5-hydroxy-*N*-methyltryptamine (5-HMeT). The homogenate was centrifuged at 16000×*g* for 10 min at 4 °C. A 20-μl aliquot of the supernatant was then analysed for biogenic amines by high performance liquid chromatography (HPLC) with electrochemical detection.

The HPLC system consisted of a Shimadzu ADVP module (Kyoto, Japan) equipped with an SIL-10 autoinjector with sample cooler and LC-10 online vacuum degassing solvent delivery unit. Chromatographic control, data collection and processing were carried out using Shimadzu Class VP data software. The mobile phase consisted of 0.1 mol phosphate buffer (pH 3.0), PIC B-8 octane sulphonic acid (0.74 mmol; Waters, Australia), sodium EDTA (0.3 mM) and methanol (12% v/v). The flow rate was maintained at 1 ml/min. Dopamine, 5-HT and 5-HMeT were separated by a Merck LiChrospher 100 RP-18 reversed phase column. Quantification was achieved via GBC LC-1210 electrochemical detector (Melbourne, Australia) equipped with a glassy carbon working electrode set at +0.75 V. The calibration curve for each standard was obtained from the concentration versus the area ratio of 50, 100 and 200 nmol standards to internal standard. Results from one PFC sample were excluded from the analysis because of suspected contamination of striatum biogenic amines, as the dopamine concentration was well above that expected in the PFC.

2.7. Statistical analyses

Power analyses were conducted using the computer program 'GPOWER' [38] for all main effects and interactions. Prior to analysis, data were assessed for linearity and homoscedasticity assumptions using the Scatterplot option and for normality assumptions using the Explore option of the Statistical Package for Social Sciences (SPSS) version 10.0 for Windows [39]. Data that were moderately positively skewed or moderately negatively skewed were transformed using square root or reflect and square root, where NEW X=SQRT ($K-X$) and K=highest value+1, transformations, respectively, prior to analysis [40].

Repeated measures analysis was performed on data collected from the same animals over time using the MIXED Model procedure SAS [41]. The covariance structure of the repeated measures data was modelled using an autoregressive with random estimates [AR(1)+RE REML] estimation [42,43]. Data were analysed for main effects of treatment and time as well as the interaction between main effects. Between-group analyses of variance (ANOVA) were carried out using SPSS Version 10.0 for Windows. Data were analysed for the main effects of treatment and delay as well as the interaction between main effects. An alpha level of .05 was used for all statistical tests.

Preliminary correlation analyses and standard multiple regression analyses of the behavioural and physical measures were conducted using SPSS Version 10 for Windows. Correlation and multiple regression analyses were conducted on a diet by delay basis. Where appropriate, correlation and multiple regression analyses were also conducted on an individual animal basis.

3. Results

3.1. Physical measurements

Digestible energy (DE) intake (kJ/g body weight) per day did not differ between treatment groups over the experimental period (Table 2). DE intake (kJ/g body weight per day) decreased significantly [$F(3,99)=6.90$, $P<.01$] over time and there was a significant interaction between diet and time [$F(6,99)=3.88$, $P<.01$] with DE intake decreasing more over time in rats consuming the SC and FC diets compared to rats consuming the Chow diet (Fig. 2).

Table 2
Mean physical parameters and S.E.D. of means over the experimental period for rats consuming a chow, soluble of FC diet

Measurement	Diet			S.E.D.	*P* values		
	Chow	SC	FC		Diet	Time	Diet× Time
Water Intake (% body weight)	22.9[a]	21.6[a]	17.5[b]	0.77	.000	.006	.000
DE Intake (kJ/g body weight)	1.42	1.34	1.40	0.04	.148	.000	.002
CP Intake (mg/g body weight)	19.2[a]	15.0[b]	15.1[b]	0.47	.000	.001	.001
Body weight (g)	238	255	255	11.3	.241	.000	.000
Faecal pH	7.22[a]	7.99[b]	7.36[a]	0.11	.000	.000	.008

Note: Means in each row with different superscripts differ significantly ($P<.05$).
S.E.D.=Standard error of difference of least squares mean; SC=soluble carbohydrate diet based on wheat; FC=fermentable carbohydrate diet based on cooked and cooled rice; DE=digestible energy calculated according to the formula used by Zijlstra et al. [76]; CP=crude protein.

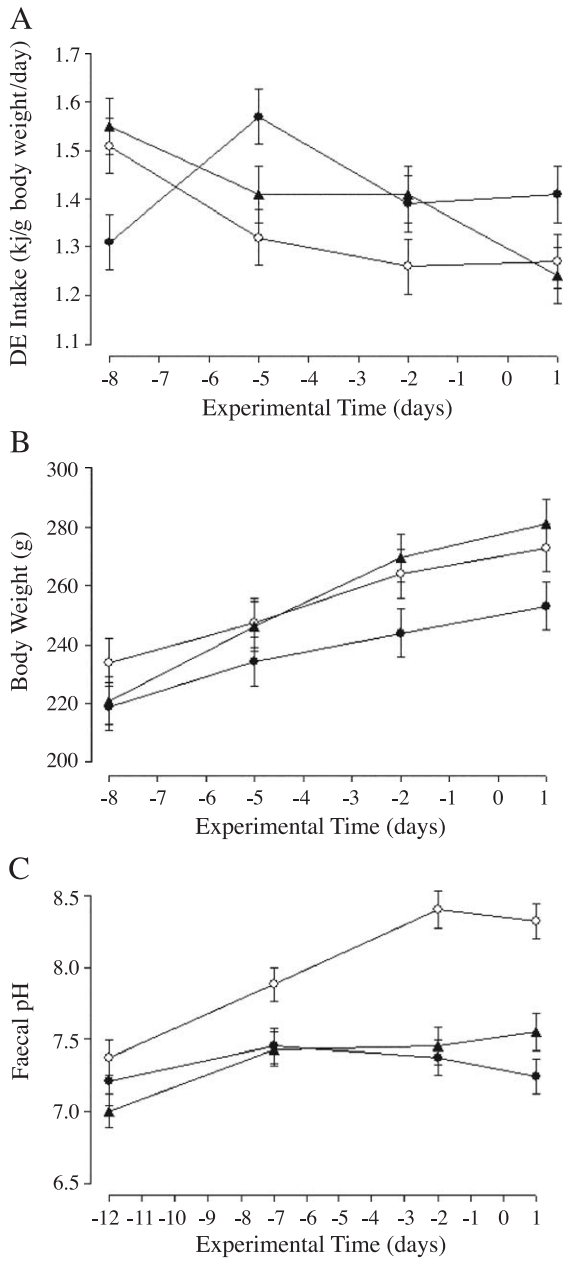

Fig. 2. DE intake (kJ/g body weight/day) (A), body weight (g) (B) and faecal pH (C) over time for rats consuming a chow (●), soluble (○) or fermentable (▲) carbohydrate diet. Error bars are standard errors of the least square means.

Faecal pH was higher in rats fed the SC diet [$F(2,33)=31.61$, $P<.01$] compared to rats fed the FC and Chow diets (Table 2). There was also a significant [$F(6,89)=3.11$, $P<.01$] interaction between diet and time indicating a change in faecal pH in rats fed the FC diet over time compared to rats fed the Chow or SC diets (Fig. 2).

Concentrations of L-lactic acid were higher [$F(2,30)=6.49$, $P<.01$] in the caecum of rats fed the FC diet compared to rats fed the Chow diet (Table 3). Concentrations of D-lactic acid were higher [$F(2,30)=5.78$, $P<.01$] in the caecum of rats fed the SC and the FC diets compared to rats fed the

Table 3
Mean pH and concentrations of lactic acid and VFA (mmol/l) in the caecum of rats at the time of sacrifice, 3 or 21 h after consuming a chow, soluble or FC diet

Measurement	3 h			21 h			S.E.D.	Diet
	Chow	SC	FC	Chow	SC	FC		*P* value
pH[#]	6.13[c]	6.35[d]	5.89[e]	6.71[a]	6.76[a]	6.50[b]	–	.000
L-lactic*	1.95[c]	3.83[b]	3.75[b]	3.77[b]	4.20[b]	6.74[a]	–	.005
D-lactic*	2.42[c]	4.73[b]	4.58[b]	4.53[b]	5.33[ab]	7.95[a]	–	.008
Acetate	84.4[e]	252.0[a]	209.5[b]	133.0[d]	177.2[bc]	162.2[cd]	15.0	.001
Propionate	12.2[d]	45.5[a]	43.7[a]	24.5[c]	29.8[bc]	34.8[b]	3.29	.000
Butyrate	21.6[c]	41.0[a]	49.2[a]	38.1[ab]	25.2[c]	30.8[bc]	5.05	.045
Total VFA	119.4[a]	343.2[b]	307.2[b]	199.8[a]	236.4[b]	231.4[b]	22.4	.000

Note: Means in each row with different superscripts differ significantly ($P<.05$).
S.E.D.=Standard error of difference of least squares mean; SC=soluble carbohydrate diet based on wheat; FC=fermentable carbohydrate diet based on cooked and cooled rice.
* Retransformed means using square root transformation prior to analysis (Units=mmol/l).
[#] Retransformed means using reflect square root transformation prior to analysis [Y=SQRT(7.55−X)].

Chow diet (refer to Table 3 for retransformed means and *P* values). Total lactic acid concentrations followed similar patterns to those described for D-lactic acid (data not shown). There were higher concentrations of L-lactic, D-lactic and total lactic acid in the caecum of rats 21 h after feeding as opposed to 3 h after feeding.

The highest [$F(2,30)=39.47$, $P<.01$] acetate concentration was found in the caecum of rats fed the SC diet, followed by the rats fed the FC diet and the lowest acetate concentration was found in the caecum of rats fed the Chow diet (Table 3). Higher concentrations of acetate [$F(1,30)=5.91$, $P<.05$] were found in the rat caecum at 3 h after feeding compared to 21 h after feeding. There was also an interaction between diet and delay [$F(2,30)=13.81$, $P<.01$] with caecal acetate concentrations highest 3 h after feeding the SC and FC diets but highest in rats fed the Chow diet 21 h after feeding. A similar diet by time interaction of caecal VFA concentration was found for propionate and butyrate. There were significantly higher concentrations of butyrate [$F(2,30)=3.44$, $P<.05$] in the caecum of rats fed the FC diet compared to rats fed the Chow diet.

Concentrations of dopamine were higher [$F(2,29)=4.04$, $P<.05$] in the PFC of rats fed the SC diet relative to rats fed the Chow and FC diets (Table 4), and there was a significant interaction between diet and delay for dopamine concentration [$F(2,29)=4.63$, $P<.05$], with a greater concentration of dopamine observed in the PFC of rats fed the SC diet at 21 h after feeding and greater concentrations of dopamine in the PFC of rats fed the Chow and FC diets at 3 h after feeding (Table 4). PFC 5-HT concentrations were not affected by diet, delay or the interaction between diet and delay.

Table 4
Mean neurotransmitter concentration (ng/g) in the PFC of rats at the time of sacrifice, 3 or 21 h after consuming a chow, soluble or FC diet

Measurement	3 h			21 h			Diet
	Chow	SC	FC	Chow	SC	FC	*P* value
Dopamine*	886.9[b]	820.6[b]	858.0[b]	461.3[b]	2244.5[a]	433.8[b]	.028
5-HT*	235.8[ab]	218.7[ab]	220.6[ab]	181.4[b]	380.3[a]	159.1[b]	.161

Note: Means in each row with different superscripts differ significantly ($P<.05$).
SC=Soluble carbohydrate diet based on wheat; FC=fermentable carbohydrate diet based on cooked and cooled rice.
* Retransformed means, transformed using square root transformation prior to analysis.

3.2. Behavioural measurements

The time spent in the light compartment of the light/dark emergence test was not affected by diet, delay and the interaction between diet and delay was also not significant (Table 5). Investigations of the unfamiliar conspecific occurred with greater frequency [$F(11,57)=2.36$, $P<.05$] when rats were fed the FC diet relative to when rats were fed the SC or Chow diets (Table 5).

The duration of play fighting with the unfamiliar conspecific was increased [$F(2,57)=6.49$, $P<.05$] when rats were fed the FC diet (M=4.11, S.D.=1.84) relative to when rats were fed the Chow diet (M=2.41, S.D.=1.68) (Table 5). A greater frequency of play fights [$F(2,57)=3.78$, $P<.05$] was initiated towards the unfamiliar conspecific by rats fed the FC (M=3.81, S.D.=1.22) and SC (M=3.68, S.D.=1.10) diets relative to rats fed the chow diet (M=2.95, S.D.=1.32). The frequency and duration of play fighting interactions made by the test rat were not affected by delay, and the interaction between diet and delay was also not significant. The duration and frequency of serious fighting interactions between the test rat and the unfamiliar conspecific were not affected by diet or delay, and there was no interactive effect of these variables.

The duration of locomotor activity was not affected by diet, delay or an interaction between diet and delay. However, the frequency of locomotor activity was higher [$F(2,63)=3.13$, $P<.05$] in rats fed the SC diet (M=164.17, S.D.=37.57) relative to rats fed the FC (M=149.52, S.D.=33.16) and Chow diets (M=139.67, S.D.=41.54). There was also an interaction between diet and delay [$F(2,63)=7.80$, $P<.01$], with locomotor activity frequency being highest in

Table 5
Mean behaviour scores in the light/dark emergence and social interaction tests for rats 3 or 21 h after consuming a chow, soluble or FC diet

Measurement	3 h			21 h			S.E.D.	Diet
	Chow	SC	FC	Chow	SC	FC		*P* value
Light time[#]	560	509	532	528	499	461	–	.622
Investigative Dur*	81.0[c]	117.5[b]	144.3[ab]	124.5[b]	164.7[a]	117.5[b]	–	.002
Investigative Freq*	55.4[b]	76.5[a]	82.0[a]	74.4[a]	77.8[a]	84.0[a]	–	.008
Play fight Dur*	3.1[b]	11.2[a]	18.0[a]	9.3[ab]	15.3[a]	16.1[a]	–	.003
Play fight Freq*	5.5[b]	12.4[a]	14.6[a]	12.6[a]	15.1[a]	14.0[a]	–	.029
Serious fight Dur*	0.34	1.24	1.06	1.38	1.59	0.79	–	.648
Serious fight Freq*	1.42	5.15	3.67	3.90	4.77	3.97	–	.261
Activity Dur	152	192	173	193	186	177	16.7	.535
Activity Freq	123	173	129	156	149	172	11.5	.051

Note: Means in each row with different superscripts differ significantly ($P<.05$).
S.E.D.=Standard error of difference of least squares mean; SC=soluble carbohydrate diet based on wheat; FC=fermentable carbohydrate diet based on cooked and cooled rice; Dur=duration (seconds); Freq=frequency; Activity=locomotor activity; SI=social interaction; Dark time=seconds.
* Retransformed means, transformed using square root transformation prior to analysis.
[#] Retransformed using reflect square root transformation prior to analysis [Y=SQRT(893.1−X)].

rats tested 21 h after feeding the Chow and FC diets but highest in rats tested 3 h after feeding the SC diet.

3.3. Correlation and multiple regression analysis

Time spent in the light compartment was negatively correlated [$r(6)=-.97$, $P<.01$] with D-lactic acid concentration in the caecum, therefore suggesting that higher concentrations of D-lactic acid in the caecum was associated with increased anxiety. None of the caecal measures or DE intake were correlated to PFC monoamine concentrations (data not shown).

Multiple regression analysis [$F(2,3)=26.95$, $P<.05$] indicated that after controlling for PFC 5-HT concentration, the amount of time spent in the light was significantly predicted by caecal D-lactic acid concentration [$sr(6)=-.97$, $P<.01$] (Table 6).

A higher concentration of 5-HT in the PFC was significantly associated with decreased rearing duration [$r(32)=-.346$, $P<.05$] and rearing frequency [$r(32)=-.356$, $P<.05$] in the social interaction test and nonsignificantly [$r(33)=-.29$, $P<.10$] with decreased activity on an individual animal basis indicating lower anxiety. A higher concentration of D-lactic [$r(34)=.372$, $P<.05$] and total lactic acid [$r(34)=.403$, $P<.05$] in the caecum was associated with increased activity frequency indicating increased anxiety. A multiple regression analysis [$F(2,30)=3.27$, $P<.05$] showed that after controlling for PFC 5-HT concentration, social interaction activity frequency of rats was significantly predicted by caecum total lactic acid concentration [$sr(32)=.393$, $P<.05$].

Play fighting frequency [$r(6)=.81$, $P=.051$] and serious fighting frequency [$r(6)=.78$, $P=.068$] were not significantly related to increased concentrations of D-lactic acid in the caecum (Table 7). Serious fighting frequency was positively correlated with concentrations of caecal acetate [$r(33)=.40$, $P<.05$], propionate [$r(33)=.44$, $P<.05$] and total VFA [$r(33)=.40$, $P<.05$] on an individual animal basis. A multiple regression analysis [$F(2,29)=3.52$, $P<.05$] (Table 8) showed

Table 6
Predictor coefficients, correlations, semipartial correlations and significance levels for a standard multiple regression with total light time[††] during the light/dark emergence test as the dependent variable and D-lactic acid and PFC 5-HT concentrations as the independent variables (Diet×Delay)

Predictor	β	Beta	r*	sr[†]	Significance
PFC 5-HT[‡]	.039	.108	.12	−.42	.485
D-Lactic acid[‡]	2.106	.992	−.97	−.97	.005

[*] Zero-order correlations.
[†] Semipartial correlations.
[‡] Transformed using square root transformation before analysis. Lactic acid units=mmol/l. 5-HT units=ng/g.
[††] Retransformed using reflect square root transformation prior to analysis [Y=SQRT(893.1−X)].

Table 7
Correlations of behavioural parameters with physical measurements (Diet×Delay)

	Caecum measure			DE
	pH[†]	D-lactic acid[‡]	Total lactic acid[‡]	
Light time[††]	.48	−.97***	−.96***	−.61
Inv duration[‡]	−.36	.51	.49	.72
Inv frequency[‡]	−.23	.87**	.86**	.79
Play fight Dur[‡]	−.10	.78	.77	.77
Play fight Freq[‡]	−.35	.81*	.80*	.82**
Serious fight Dur[‡]	−.62	.54	.53	.63
Serious fight Freq[‡]	−.48	.78*	.77*	.57
Activity duration	−.62	.51	.50	.45
Activity frequency	−.50	.68	.68	.22

DE=Digestible energy intake calculated according to the formula used by Zijlstra et al. [76]; SI=social interaction; dark time=seconds.
* $P<.07$, two tailed.
** $P<.05$, two tailed.
*** $P<.01$, two tailed.
[†] Transformed using reflect square root transformation before analysis [Y=SQRT(7.55−X)].
[‡] Transformed using square root transformation before analysis (lactic acid units=mmol/l).
[††] Retransformed using reflect square root transformation prior to analysis [Y=SQRT(893.1−X)].

Table 8
Predictor coefficients, correlations, semipartial correlations and significance levels for a standard multiple regression with serious fighting frequency as the dependent variable and caecum propionate and PFC dopamine concentrations as the independent variables (individual animal)

Predictor	β	Beta	r*	$sr^{\dagger}$	Significance
PFC dopamine‡	.000007	.005	−.03	.01	.98
Caecum propionate‡	.01533	.442	.44	.44	.01

* Zero-order correlations.
† Semipartial correlations.
‡ Transformed using square root transformation before analysis. Propionate units=mmol/l. Dopamine units=ng/g.

that after controlling for PFC dopamine concentration, the serious fighting frequency of rats was significantly predicted by caecum propionate concentration [$sr(31)=.44$, $P<.05$].

4. Discussion

The present study supports the hypothesis that diets differing in carbohydrate components that alter fermentation in the hindgut are associated with behavioural disturbances in rats. Supporting this notion was the finding that the amount of time spent in the light compartment during the light/dark emergence test decreased as concentrations of D- and total lactic acid in the caecum increased. In addition, standard multiple regression analysis revealed that the total time spent in the light was significantly predicted by D-lactic acid, as opposed to PFC 5-HT concentrations. Our results agree with those presented by Gurtman et al. [44], in that less time was spent in the light compartment as anxiety increased [36], suggesting that fermentation and increased lactic acid concentrations in the caecum of rats are associated with increased anxiety.

Although the negative association between PFC 5-HT concentration and locomotor activity was not significant, increased locomotion is generally associated with increased anxiety responses in rats [45,46]. Because 5-HT is well regarded as having a calming effect on animals [47] and serotonin specific reuptake inhibitors (SSRIs) that selectively increase 5-HT concentrations are commonly used to treat a number of anxiety disorders [48], it appears therefore that an increased activity duration and frequency in the social interaction test also indicates increased anxiety. An increased concentration of lactic acid in the caecum of rats was also related to an increase in locomotor activity frequency after controlling for PFC 5-HT, therefore indicating increased anxiety. The present results suggest that the relationship between 5-HT and feelings of calmness in humans [49] and animals [50] may also be altered, at least in part, by changes in fermentation and lactic acid.

Increased occurrence of aggression in rats has previously been linked to higher concentrations of dopamine [51,52]. Serious fighting frequency in the current study was significantly predicted by caecal propionate concentration after controlling for PFC dopamine concentration. Although dopamine concentrations in the PFC appeared to be more variable than those observed previously [53] and may need to be repeated in future studies, these results agree with previous findings that propionate is an important VFA associated with adverse behaviour in horses [54]. Although the frequency of playful and serious fights initiated by the test rats towards the unfamiliar conspecific was not significantly related to concentrations of D- and total lactic acid in the caecum, the occurrence of playful fighting in young rats can also be indicative of the development of more serious fighting later in life [55,56]. A larger sample size than that used in the present study, or the study of older rats, may be necessary to resolve the issue as to whether serious fighting can be significantly predicted by higher concentrations of lactic acid.

D-Lactic acid has been thought to be the most detrimental short-chain monocarboxylic acid produced from fermentation of carbohydrate in the gut as it can produce numerous negative physical, mood and behavioural effects in humans [26] and animals (for a review, see Refs. [43,57]). This hypothesis was not supported by the current study, which showed that behaviours indicative of increased anxiety and aggression were significantly correlated with both D- and L-lactic acid and propionate concentrations in the caecum.

The mechanism of action leading to increased anxiety following intravenous infusions of D- or L-lactic acid remains unclear [58,59]. There may be differences in the capacity of each isomer of lactic acid to initiate anxiety as D-lactic acid appears to be absorbed across the blood brain barrier by passive diffusion, in contrast to L-lactic acid, which has a higher affinity to the monocarboxylic acid carrier-mediated transport system [60,61]. Alterations in brain uptake of lactic acid may depend on relative concentrations of each isomer or plasma pH. Alterations in lactic acid transport into the brain may be associated with several effects. For example, it has been suggested that D-lactic acid may be directly neurotoxic [62,63]; however, it is more likely that D- and L-lactic acid may affect the metabolism of neurotransmitters, either directly or through alterations to intra- or extracellular pH.

Data from the present study suggest a possible role of 5-HT alterations in the PFC in anxiety. Further analyses of changes in dopamine and 5-HT concentrations in the amygdala as well as lactic acid in brain regions associated with anxiety and aggression should be undertaken following dietary manipulation.

The results of the present study have several implications both in research and in clinical practice. For example, comparisons of behavioural characteristics between animal studies may sometimes be confounded by differences in diet instead of, or in addition to, differences related to the treatments under investigation. The majority of research using rat subjects merely states that a standard laboratory chow was used ad libitum and researchers do not specify an analysis of the diet's constituents. Anxious behaviour

that is promoted by diet, for example, may mask the effect of an anxiolytic treatment. Conversely, pharmacological intervention that promotes an alteration in gut rate of passage may change digestion and absorption parameters leading to changes in the pattern of fermentation in the hindgut altering the responses produced by the pharmacological intervention.

Increased aggression and anxiety are common presenting problems of clients attending many clinical psychology services and are considered to be caused by learned behaviour and thinking styles and neurochemical alterations. Alteration of diet is rarely considered in clinical practice for treatment of these two types of behaviour; however, this approach could be more beneficial than pharmacological treatments [30,64] that may have more side effects.

Recently, human nutritionists have encouraged the consumption of foods containing resistant starch and nonstarch polysaccharides (e.g., see Refs. [65,66]), as increased butyrate production from fermentation of these components in the caecum and colon are thought to decrease the risk of colon cancer [67,68]. However, there are a number of methodological problems in this area of research, as only the in vitro effects of butyrate alone have been studied without examining combinations containing other fermentation end products such as acetate, propionate and lactic acid [70]. Acetic acid, for example, can induce ulcerative colitis [71,72]. There is also no in vivo evidence showing preventative effects of increasing fermentation and butyrate on colonic cancer [69], and some research indicates that butyrate can increase cell proliferation in some cell lines [67,73,74]. Lastly, and perhaps more importantly, no research has been conducted into the possible negative effects on human behaviour of this increased hindgut fermentation.

Future work needs to establish whether increased fermentation and lactic acid is the cause of the observed increase in anxiety and aggression. Empirical studies need to elucidate whether causal mechanisms linking lactic acid and VFAs with anxiety and aggression exist by examining mechanisms of absorption, metabolism, transport through the blood brain barrier and neurochemistry alterations, such as 5-HT functioning, in various brain regions following increased hindgut fermentation. These mechanisms can only be confirmed after examining whether the alterations in behaviour can be attenuated using the same diets but preventing lactic acid accumulation or blocking the action of lactic acid at any of the steps indicated above [75].

In conclusion, increased fermentation with increased concentrations of lactic acid and VFAs in the hindgut of rats is associated with increased anxious and aggressive behaviour. The current rat model of acidosis appears to be a valid and reliable model to use for human research and has direct clinical implications. Researchers need to consider diet as an important methodological consideration, which can interact with a number of variables under investigation. The study of the mechanism of action leading to nutritional effects on anxiety and aggression warrants further empirical and clinical research.

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