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Increased D-Lactic Acid Intestinal Bacteria in Patients with Chronic Fatigue Syndrome

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Abstract. *Patients with chronic fatigue syndrome (CFS) are affected by symptoms of cognitive dysfunction and neurological impairment, the cause of which has yet to be elucidated. However, these symptoms are strikingly similar to those of patients presented with D-lactic acidosis. A significant increase of Gram positive facultative anaerobic faecal microorganisms in 108 CFS patients as compared to 177 control subjects ($p<0.01$) is presented in this report. The viable count of D-lactic acid producing Enterococcus and Streptococcus spp. in the faecal samples from the CFS group (3.5×10^7 cfu/L and 9.8×10^7 cfu/L respectively) were significantly higher than those for the control group (5.0×10^6 cfu/L and 8.9×10^4 cfu/L respectively). Analysis of exo-metabolic profiles of Enterococcus faecalis and Streptococcus sanguinis, representatives of Enterococcus and Streptococcus spp. respectively, by NMR and HPLC showed that these organisms produced significantly more lactic acid ($p<0.01$) from ^{13}C-labeled glucose, than the Gram negative Escherichia coli. Further, both E. faecalis and S. sanguinis secrete more D-lactic acid than E. coli. This study suggests a probable link between intestinal colonization of Gram positive facultative anaerobic D-lactic acid bacteria and symptom expressions in a subgroup of patients with CFS. Given the fact that this might explain not only neurocognitive dysfunction in CFS patients but also mitochondrial dysfunction, these findings may have important clinical implications.*

Chronic fatigue syndrome (CFS) is a debilitating condition where patients report severe symptoms including protracted fatigue, immune alterations and musculoskeletal pain (1, 2). These prime symptoms are frequently exacerbated by cognitive and neurological impairments and gastrointestinal dysfunction (3). Cognitive and neurological impairments have been reported in patients with elevated blood lactic acid (4). In a review of 29 patients with D-lactic acidosis, all were reported to have mild to severe cognitive dysfunction with varying neurological impairment including dysarthria, ataxia, weakness and inability to concentrate (5). D-lactic acidosis is a metabolic condition that originates from the bacterial fermentation of carbohydrates by gastrointestinal (GI) bacteria (6). The elevated blood lactic acid seen in these patients was shown to be related to an overgrowth of colonic Gram positive anaerobic lactic acid bacteria, including *Lactobacillus* (4, 7), *Bifidobacterium* (8). However, other facultative anaerobic lactic acid bacteria, including *Enterococcus* and *Streptococcus* spp. were not looked for in these studies.

Lactic acid bacteria can be classified either homo- or heterofermentative according to their glucose catabolism in the Embden-Meyerhof-Parnas pathway. Both *Enterococcus* and *Streptococcus* spp. are classified as homofermentative, producing only lactic acid from glucose catabolism and generally regarded as potent D- and L-lactic acid producers. Members of the genus *Lactobacillus* and *Bifidobacterium* are classified as heterofermentative, producing ethanol and carbon dioxide in addition to lactic acid from glucose.

Nuclear magnetic resonance (NMR) exo-metabolic profiling is a robust technique for obtaining a metabolic fingerprint of bacteria by detecting end products of microbial

Abbreviations: Chronic fatigue syndrome (CFS); gastrointestinal (GI); high performance liquid chromatography (HPLC); nuclear magnetic resonance (NMR); short bowel syndrome (SBS).

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metabolism and the rapid quantitative determination of concentrations of microbial metabolites (9, 10) . To accompany the detailed NMR exo-metabolome profiling, high pressure liquid chromatography (HPLC) is widely used for targeted metabolite profiling (11).

The gastrointestinal and particularly the colonic microbial ecosystem of chronically ill patients has been poorly investigated in the past and little or no data of the faecal microbial metabolic flux of CFS patients has been reported. The aim of this study is two-fold. Firstly, to determine any significant relationship between the colonization of homofermentative D- and L-lactic acid bacteria in the gastrointestinal tract of CFS patients. Secondly, to investigate the metabolic profiles of lactic acid bacteria identified in faecal samples from CFS patients using NMR spectroscopy and HPLC.

Materials and Methods

Study population. A retrospective study of faecal microbial analysis from CFS patients, examined and cared for by one investigator (DPL), was conducted during the period 2005-2006. A total of 108 patients were included in the study. The diagnosis of CFS was based upon the clinical and chronological history, the physical examination and the investigations that were performed. These steps are in accordance with the Guidelines for the Diagnosis of CFS proposed by the RACP (12). The patients fulfilled the Holmes (13) /Fukuda (14) / Canadian Definition Criteria (15). The decision to proceed with faecal analysis was determined by the presence of gastrointestinal symptoms in those who were refractory to other treatment modalities. The patients were provided with a questionnaire addressing symptom incidence and severity (16). The symptom scores were graded from '0' (no effect) to '4' (severe effect) relating to how much a patient had been affected by each of the 86 symptoms in the past seven days prior to sampling. The faecal microbial analysis of a further 177 subjects, all self reported with little or no cognitive and neurological impairment (mind fogginess, slurred speech, trouble remembering things, ataxia, weakness and inability to concentrate) were included as controls for comparison.

Faecal sample collection. All patients and control subjects were instructed to avoid all antimicrobial agents three weeks prior to the collection of a faecal sample. The first morning bowel motion was collected in a faecal container and immediately transported in a sealed anaerobic pouch system (Oxoid, Adelaide, Australia). The lid of the faecal container was modified and perforated to assist sample anaerobiasis. Anaerobiasis was achieved by activating the Anaero Gen Compact (Oxoid, Adelaide, Australia) prior to the pouch being sealed. Samples were transported cold (<12˚C) to the laboratory and analyzed within 48h after collection. Samples were rejected for analysis if they were delayed during transit, had inadequate refrigeration or if the anaerobic incubation during transport did not meet the criteria. Samples from patients taking antimicrobial agents during the three week period immediately prior to specimen collection were also rejected.

Quantitation and identification of faecal microorganisms. All faecal samples, once removed from the anaerobic pouch system, were processed within 10-15 min. A determined quantity of faecal sample (range 0.5-1.0 g) was transferred to 10mL of 1% glucose-saline buffer (17). Hundred and thousand fold serial dilutions (beginning from 10^{-1} to 10^{-7}) of homogenized faecal samples were prepared. A 10 and 1 μL amount of each dilution was transferred onto previously dried horse blood agar (Oxoid), chromogenic medium (Oxoid) colistin and nalidixic acid blood agar (Oxoid) for aerobic incubation. All media were incubated at 35˚C for 48h.

To determine the validity of the anaerobic transport method, two faecal samples from one of the investigators (HLB), collected at different days, were processed each within two hours after collection and again on two different occasions, at 24 and 48h. On each occasion the specimen was re-sealed and stored refrigerated anaerobically immediately after processed. Results from this internal quality assurance investigation showed that there were no significant quantitative changes in either the facultative aerobes or anaerobes processed at the three different periods. The incidence of the predominant facultative aerobes and anaerobes remained unchanged.

Facultative anaerobic organisms were identified using standard criteria (18). In brief, aerobic Gram positive cocci with a negative catalase reaction grown in 6.5% NaCl and positive reaction on bile-esculin medium were identified as *Enterococcus* spp. Organisms with a positive β-D galactosidase reaction on chromogenic medium and which produced indole were identified as *Escherichia coli*. Coliform-like organisms that gave a negative β-D galactosidase and a positive β-glucosidase reaction on the chromogenic medium were further identified using the MB12A (Microbact) system.

Three organisms were used for the metabolic profile study. These were *Escherichia coli* (ATCC 25922) and two clinical faecal isolates, *Enterococcus faecalis* (*E. faecalis*) and *Streptococcus sanguinis* (*S. sanguinis*). Both clinical isolates were identified using the RapID STR (Remel) system.

Preparation of ^{13}C labeled bacterial exo-metabolites for HPLC. A sterilized 0.2% ^{13}C D-glucose (Sigma) solution in Luria broth (tryptone (Sigma) 10g, NaCl (Sigma) 10g, yeast extract (Sigma) 5g, RO water 1L) was prepared. A 100μL amount of each of the three organisms for metabolic profile study (*E. coli* (ATCC 25922), *E. faecalis* and *S. sanguinis*) in sterile saline were added to 10mL of LB broth (n=5) and incubated at 36˚C at time intervals of 4, 8 and 16 hours. At the end of each incubation, a 10 mL fraction was snap-frozen and lyophilised overnight before re-suspended in 1 mL of 18 mΩ water to be analysed by HPLC. Sterile LB broth was used as control.

Preparation of ^{13}C labeled bacteria samples for NMR and pH analysis. 45 mL sterile stock solutions of 100 mg/L ^{13}C-glucose (Spectral Gases Laboratories) in Brain Heart Infusion (Oxoid) were prepared and inoculated with 10 μL of either *E. coli*, *E. faecalis* or *S. sanguinis*. Each of the three stock solutions was further divided into 15 mL working solutions, with a final concentration of 1.45 % 13C-glucose. The working solutions were incubated at 36˚C at time intervals of 2, 4, 6, 8, 10, 12, 14 and 16 h (n=5 for each bacteria strain and each incubation period). A sterile working solution was used as control. Following each incubation, working solutions were centrifuged at 5000 g at 4˚C for 20 minutes and 4mL of the supernatant removed and lyophilised overnight. The lyophilised fractions were re-suspended in 550 μL of D_2O (containing 0.02% w/v NaN_3), adjusted to pH 7.0 with DCl or NaOD and placed in 5 mm NMR tubes.

The bacterial supernatant pH of each incubation was determined by a pH 211 Microprocessor (Hanna Instruments).

Table I. *Mean viable counts (cfu/g) of faecal aerobes from CFS patients (n=108) and control subjects (n=177).*

Organisms	Control	CFS patients	*P*-value
Total aerobes	1.09×10^8	1.93×10^8	$p<0.001$
Gram negative to Gram positive ratio	9742.7	238.9	$p<0.01$
E.coli	1.0×10^8	4.26×10^7	$p=0.98$
E. faecalis	5.0×10^6	3.5×10^7	$p<0.001$
S. sanguinis	8.9×10^4	9.8×10^7	$p<0.001$

NMR analysis. Spectra were acquired using a Bruker Avance 800 MHz spectrometer with a 5 mm triple resonance cryoprobe. All samples were locked to D_2O and chemical shift referenced to lactate at 1.31 ppm. ^1H – ^{13}C Heteronuclear Single Quantum Coherence (HSQC) was acquired on the bacterial samples (19, 20). The data was acquired using echo/anti-echo gradient selection, Gaussian shaped pulses (21) for all ^{13}C 180˚ pulses and ^{13}C decoupling applied during acquisition. The 90˚ pulse for ^1H was 7.8 μs, and for ^{13}C was 15.0 μs. The spectral widths for ^1H and ^{13}C were 13 ppm and 50 ppm with 256 transients collected into 2048 data points in the time domain followed by a 1 s relaxation delay. The first row Free Induction Decay (FID) data was extracted to yield a ^{13}C-filtered one-dimensional spectrum so only 1H resonances attached to ^{13}C were resolved. The first row FIDs were processed with a squared cosine bell window function.

Chiral HPLC analysis. The re-suspended bacterial fractions in ^{13}C-glucose LB broth were analysed on an Agilent 1200 series HPLC system equipped with a Phenomenex Chirex 3126 (D) Penacillamine, 150×4.60 mm, 5 micron chiral column. The L- and D- lactic acids were eluted under isocratic mobile phase conditions of 2 mM copper sulphate containing 5% isopropanol with a flow rate of 1.0 mL min^{-1} Detection was determined at 254 nm.

Statistical analysis. All microbiology data were log10 transformed prior to statistical analysis. Group differences were assessed by Students *t*-tests. These data were processed using Statistica™ (Ver. 5.5, Statsoft, Tulsa). Metabolites detected by NMR were analysed using one way ANOVA (Analysis of Variance) *via* Prism 4.0 (GraphPad) and Bonferroni two-tailed paired *t*-tests with $p<0.05$ taken to be statistically significant. The results were expressed in bar graphs as mean±standard error of the mean for the concentrations of the total number of observations over each variable for each experiment. Automated non-linear regression models were fitted to the data for lactate concentration curves to illustrate the trajectory of lactate secretion by *E. coli*, *E. faecalis* and *S. sanguinis*.

Results

Faecal microbial flora of CFS patients and control subjects. The mean viable count of the total aerobic microbial flora for the CFS group (1.93×10^8 cfu/g) was significantly higher than the control group (1.09×10^8 cfu/g) ($p<0.001$). There was a significant predominance of Gram positive aerobic organisms in the faecal microbial flora of CFS patients. The ratio between the aerobic Gram negative and Gram positive organisms for CFS patients (mean 238.9) was significantly lower than the control subjects (mean 9742.7) ($p<0.01$). The viable count for the aerobic organism *E. coli* between the two groups did not differ significantly ($p=0.98$, Table I). Comparatively, the mean counts for the organisms *E. faecalis* and *S. sanguinis* for the CFS group (3.5×10^7 cfu/L and 9.8×10^7 cfu/L respectively) were significantly higher than that for the control group (5.0×10^6 cfu/L and 8.9×10^4 cfu/L respectively) ($p<0.001$, Table I).

Exometabolic flux analysis by NMR and HPLC. ^{13}C-edited NMR spectra were used to monitor the metabolic efflux of *E. faecalis*, *S. sanguinis* and *E. coli* over a 16 h time period. Spectral analysis showed the predominant metabolites of *E. coli* from glucose catabolism were lactic acid, acetic acid and succinic acid (Figure 1). By contrast the predominant metabolites of *E. faecalis* and *S. sanguinis* were lactic acid with a trace of acetic acid (Figure 1). Analysis by two-tailed paired *t*-test showed that *E. faecalis* and *S. sanguinis* produced significantly more lactic acid than *E. coli* during the 16 h growth period ($p<0.001$) (Figure 2).

The pH for all three bacteria strains over the incubation phases ($p<0.001$) was acidic (Figure 3). *E. faecalis* and *S. sanguinis* maintained average pH values of 4.34 and 4.45 respectively, while *E. coli* showed an average pH of 5.3. This observation is consistent with the higher lactate production of *E. faecalis* and *S. sanguinis*.

D- and L-lactic acid isomers were separated by chiral-HPLC. *E. coli* produced predominantly L-lactic acid. In contrast *E. faecalis* and *S. sanguinis* produced more D-lactic acid over a 16 h incubation (Figure 4).

Discussion

This study confirms the previous observation (22), and those reported by other investigators (23) that there was a marked alteration of faecal microbial flora in a sub-group of CFS patients. The study shows that there is an overall increase of aerobic Gram positive intestinal bacteria particularly those of the *Enterococcus* and *Streptococcus* spp. Furthermore, exometabolic footprinting by NMR and HPLC showed that representatives of these homofermentative microflora produce

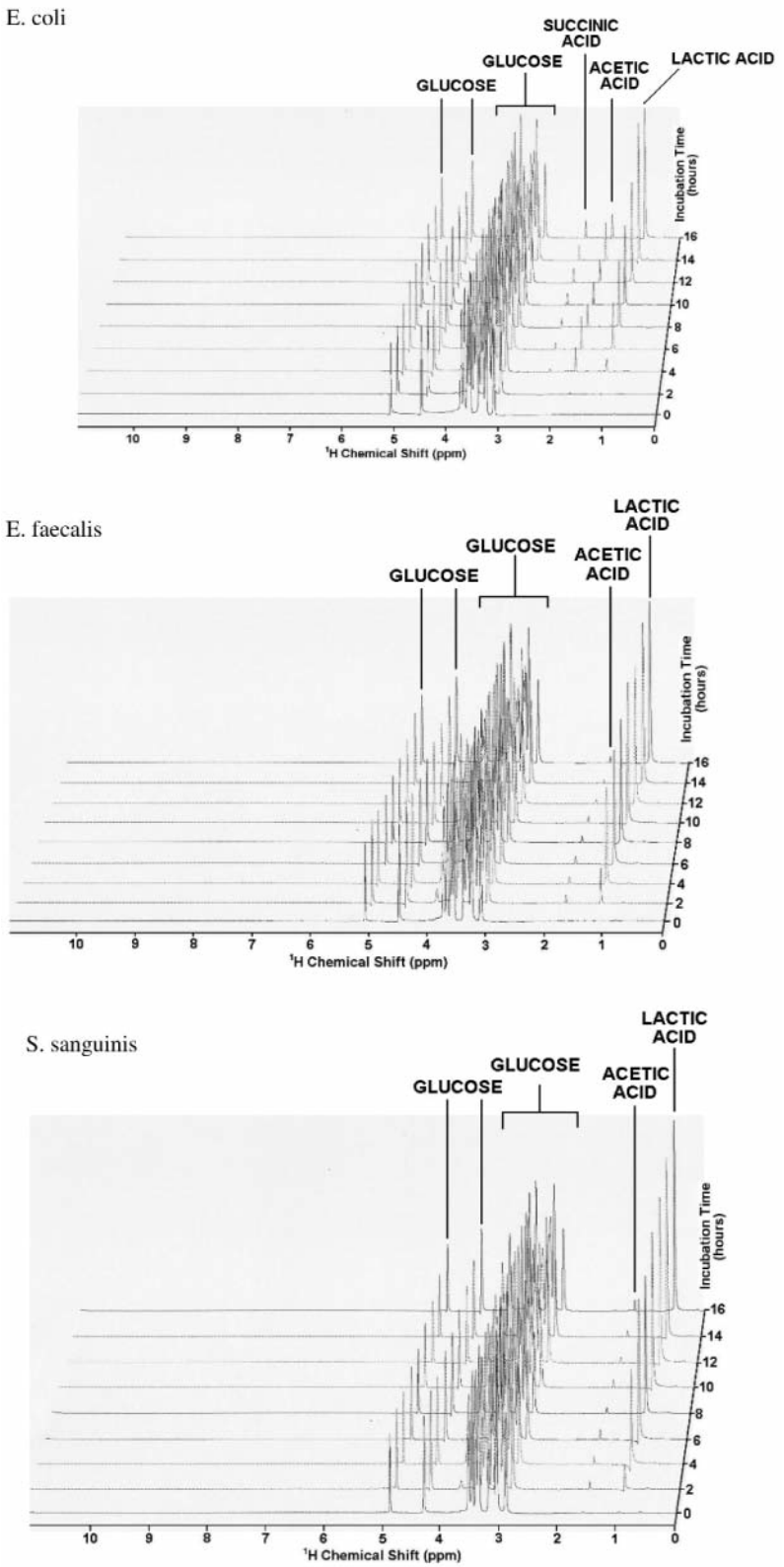

Figure 1. *^{1}H-^{13}C edited HSQC first row 1D NMR spectral time plots for E. coli, E. faecalis and S. sanguinis respectively, incubated over a 16 hour period. These profiles identify lactic acid (1.32 and 4.10 ppm), acetic acid (1.91 ppm) and succinic acid (2.40 ppm) as exometabolites secreted into the growth medium from the metabolism of ^{13}C-glucose.*

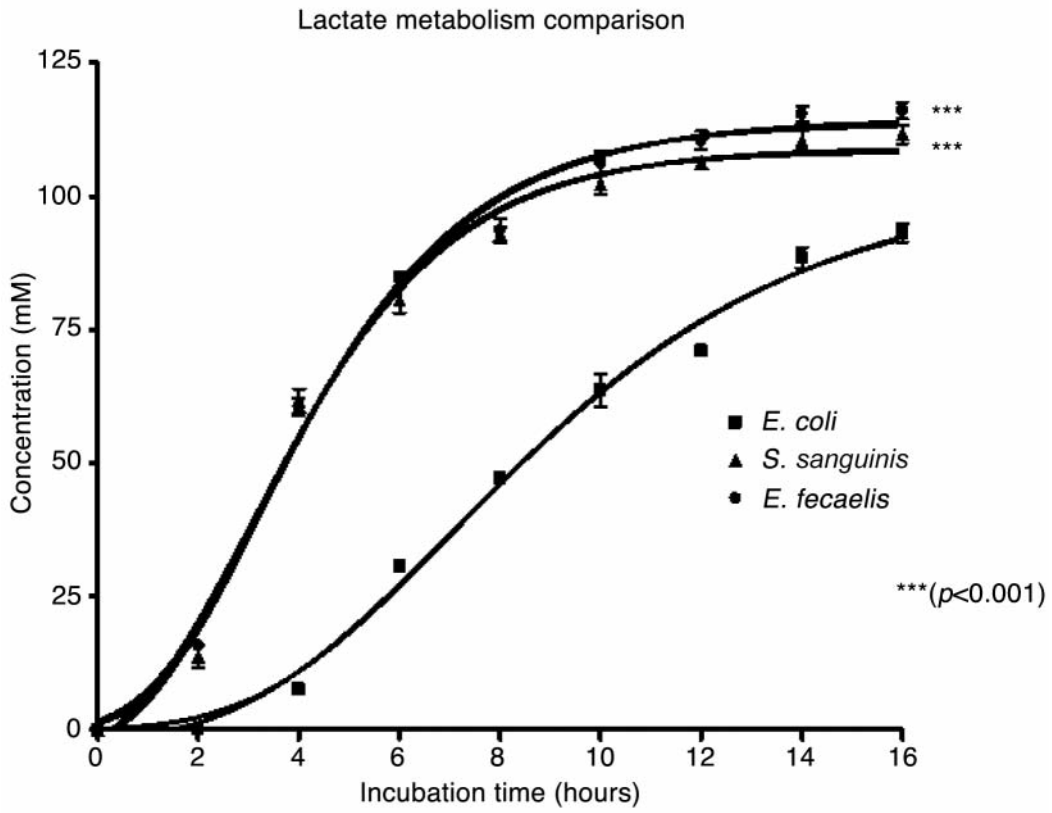

Figure 2. *Comparison of ^{13}C-lactic acid secretion by E. coli, E. faecalis and S. sanguinis over a 16 hour incubation period as determined by NMR. The two Gram positive anaerobes (organisms E. faecalis and S. sanguinis) secrete twice as much lactic acid than E. coli at the growth phase (6 hours) and significantly more lactic acid than E. coli at 16 hours.*

D- and L-lactic acids from glucose metabolism. D-lactic acid, if found elevated in serum, has been reported to be associated with cognitive dysfunction and neurological impairment in patients with intestinal bacterial overgrowth (5, 24).

The large intestine is populated with an excess of 10^{11}-10^{12} bacteria per gram of faecal material and comprising over 500 different bacterial species (25). In a study of 12 healthy men aged between 20-60yr, the total viable bacterial count in faeces for aerobes was estimated to be between 106 and 108 cfu/g (26). The organisms *E. coli* and *Enterococcus* spp. are the most common aerobic bacteria isolated from the gastrointestinal tract of humans, with *E. coli* being the most predominant species, occupying 90-95% and *Enterococcus* spp 1-10% of the total aerobic intestinal flora (27). The organism *Enterococcus* is generally regarded as an opportunistic invader of the intestine and is suppressed to a low level during development of the adult flora (28). Noble (29) examined the carriage incidence of group D *Streptococcus* in human adult faeces and found faecal content of the organisms to be 10^5 to 10^7 cfu/g of stool. Benno (30) compared the faecal microflora of rural Japanese with urban Canadians and found that 100% of both groups had the organism *Enterococcus* and that the organism was present at about $10^{6.5}$ cfu/g. Similarly Ducluzeau (27) examined the microbial ecology of the human intestine and found that faecal *Streptococcus* had a count of 10^6 to 10^7 cfu/g, which represented 1-10% of the total aerobic intestinal flora. In this study the mean total count for *Enterococcus* and *Streptococcus* spp. for the CFS group was 52% of the total aerobic intestinal flora, which is significantly higher than the 12% seen in the control subjects (p<0.01).

Patients with CFS present remarkably similar symptoms to patients with D-lactic acidosis including headaches, weakness, cognitive impairment, fatigue, pain and severe lethargy (5, 7, 24, 31). These severe cognitive and neurological impairments, seen in patients with D-lactic acidosis, are thought to be attributed to the increased colonization of lactic acid producing Gram positive bacteria in the gastrointestinal tract (32, 33). High count of lactic acid bacteria in the gastrointestinal tract may lower intestinal pH levels resulting intestinal permeability (34), and may perpetuate an increased absorption of microbial exometabolites such as D-lactic acid. D-lactic acid, unlike L-lactic acid, is poorly metabolized in mammalian hosts as mammals lack the metabolic enzyme D-lactate-dehydrogenase (35, 36). Increased intestinal colonization of D-lactic acid

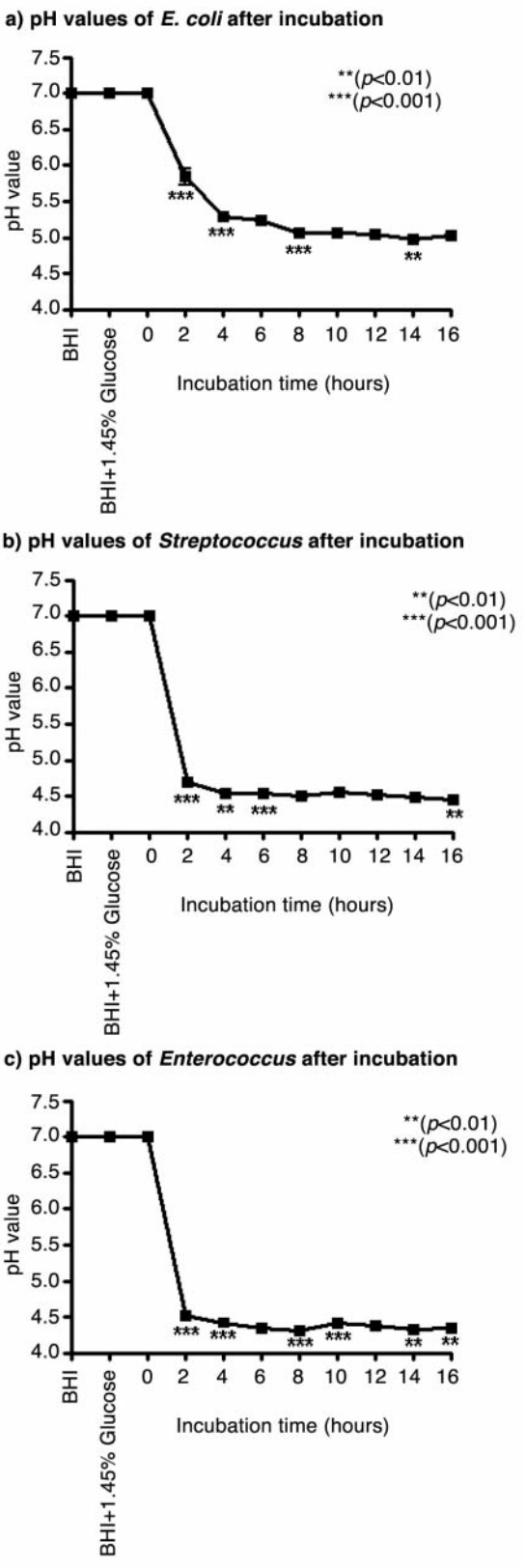

Figure 3. *Comparison of pH values taken from the media of E. coli (A), E. faecalis (B) and S. sanguinis (C) over 16 hours (n=4).*

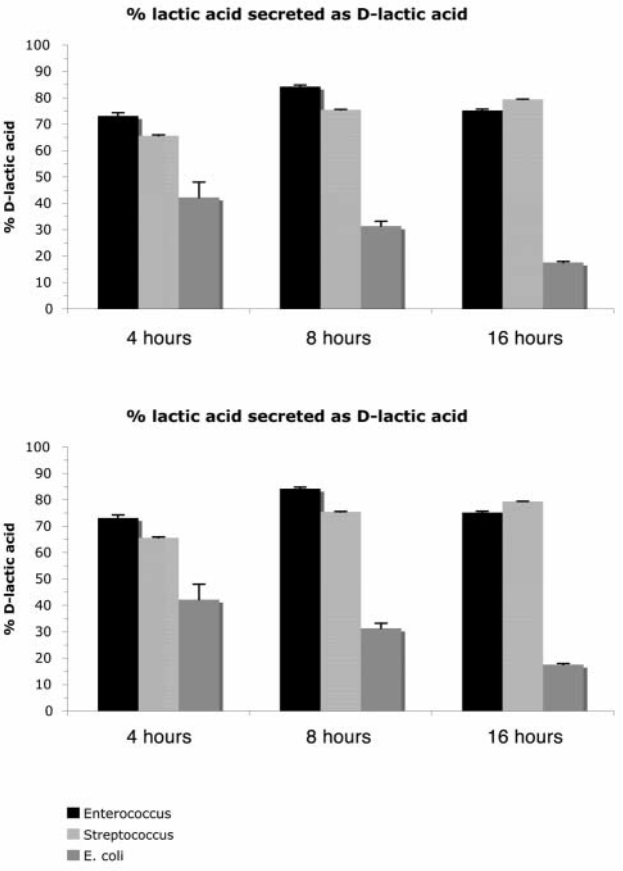

Figure 4. *Chiral-HPLC peak areas were used to calculate the percentage of lactic acid existing as D-lactic acid that was produced by each microorganisms at the times shown (n=4).*

producing bacteria, as demonstrated in this study, may result in pathophysiological cognitive and neurological responses in CFS patients as reported in patients with D-lactic acidosis.

In this study the NMR-based metabolic profiles of the three intestinal micro-organisms, *E. faecalis*., *S. sanguinis*. and *E. coli* showed that the Gram positive bacteria (*Enterococcus* and *Streptococcus* spp.) produce more lactic acid than the Gram negative *E. coli*. Not surprisingly, these Gram positive bacteria were shown to lower the ambient pH of their environment *in vitro* as compared to that of *E. coli*. This suggests that when *Enterococcus* and *Streptococcus* spp. colonization in the intestinal tract is increased, the heightened intestinal permeability caused by increased lactic acid production may facilitate higher absorption of D-lactic acid into the bloodstream, henceforth perpetuating the symptoms of D-lactic acidosis. Increased intestinal permeability is also associated with endotoxin release from Gram negative enterobacteria, leading to inflammation, immune activation and oxidative stress, which are cardinal features in a large subset of CFS patients (37).

Indigenous bowel microflora can have both positive and negative impacts on health. The balance of this ecosystem is dynamic and alteration of intestinal bacteria may alter the nutritional benefits to the host. Results in this study elucidate a direct relationship between CFS patients with cognitive dysfunction and marked alteration of the bowel microbial flora where the dominance of the organism *E. coli* was replaced by the Gram positive homofermentative lactic acid bacteria, *Enterococcus* and *Streptococcus* spp. This relationship demonstrates the need for measuring D- and L-lactic acid accumulation in biofluids of CFS patients compared to healthy individuals to confirm whether D-lactic acid may be involved in the pathogenesis of CFS.

The cause for the increased colonization of faecal enterococcus and streptococcus in patients with CFS remains unclear. A recent report of persistent enterovirus infection in the intestinal tract of CFS patients suggests the possibility of a chronic disseminated enteroviral infection disrupting the local immunity (38). Microbial translocation is also a cause of systemic immune activation in HIV infected individuals (39). Based on the findings described in this study, existing therapeutic tools such as short courses of an appropriate antibiotic, alkalinizing agents, a low carbohydrate diet and the restriction of glucose intake could become part of the therapy of CFS patients who suffer from D-lactic acidosis.

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References

1 Hudson JI, Goldenberg DL, Pope HG Jr, Keck PE Jr and Schlesinger L: Comorbidity of fibromyalgia with medical and psychiatric disorders. Am J Med *92*: 363-367, 1992.

2 Gomborone JE, Gorard DA, Dewsnap PA, Libby GW and Farthing MJ: Prevalence of irritable bowel syndrome in chronic fatigue. J R Coll Physicians Lond *30*: 512-513, 1996.

3 McGregor NR, Dunstan RH, Zerbes M, Butt HL, Roberts TK and Klineberg IJ: Preliminary determiantion of a molecular basis to chronic fatigue syndorme. Biochem Mol Med *57*: 73-80, 1996.

4 Caldarini MI, Pons S, D'Agostino D, DePaula JA, Greco G, Negri G, Ascione A and Bustos D: Abnormal fecal flora in a patient with short bowel syndrome. An *in vitro* study on effect of pH on D-lactic acid production. Dig Dis Sci *41*: 1649-1652, 1996.

5 Uribarri J, Oh MS and Carroll HJ: D-lactic acidosis. A review of clinical presentation, biochemical features, and pathophysiologic mechanisms. Medicine (Baltimore) *77*: 73-82, 1998.

6 Shah M, Beuerlein M and Danayan K: An approach to the patient with a life-threatening acid-base disturbance: The acidemias. University of Toronto Medical Journal *78*: 122-128, 2001.

7 Hove H and Mortensen PB: Colonic lactate metabolism and D-lactic acidosis. Dig Dis Sci *40*: 320-330, 1995.

8 Kaneko T, Bando Y, Kurihara H, Satomi K, Nonoyama K and Matsuura N: Fecal microflora in a patient with short-bowel syndrome and identification of dominant lactobacilli. J Clin Microbiol *35*: 3181-3185, 1997.

9 Ramos A, Rute Neves A and Santos H: Metabolism of lactic acid bacteria studied by nuclear magnetic resonance. Antonie van Leeuwenhoek *82*: 249-261, 2002.

10 Seop Kim I, Barrow KD and Rogers PL: Application of nuclear magnetic resonance spectroscopy to analysis of ethanol fermentation kinetics in yeasts and bacteria. Biotechnol Letters *21*: 839-848, 1999.

11 Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG and Kell DB: Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol *22*: 245-252, 2004.

12 Working Group of the Royal Australasian College of Physicians. Chronic fatigue syndrome. Clinical practice guidelines 2002. Med J Aust *76*: S23-56, 2002.

13 Holmes GP, Kaplan JE, Gantz NM, Komaroff AL, Schonberger LB, Straus SE, Jones JF, Dubois RE, Cunningham-Rundles C and Pahwa S: Chronic fatigue syndrome: a working case definition. Ann Intern Med *108*: 387-389, 1988.

14 Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG and Komaroff A: The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Ann Intern Med *121*: 953-959, 1994.

15 Carruthers BM, Jain AK, De Meirleir K, Peterson DL, Klimas NG, Lerner AM, Bested AC, Pierre FH, Joshi P, Powles ACP, Sherkey JA and van de Sande MI: Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome: Clinical Working Case Definition, Diagnostic and Treatment Guidelines. A Consensus Document. J Chronic Fatigue Syndr *11*: 7-115, 2003.

16 McGregor NR, Dunstan RH, Zerbes M, Butt HL, Roberts TK and Klineberg IJ: Preliminary determination of the association between symptom expression and urinary metabolities in subjects with chronic fatigue syndrome. Biochem Mol Med *58*: 85-92, 1996.

17 Willis AT: Anaerobic culture methods. *In*: Levett PN, ed. Anaerobic Microbiology: A Practical Approach. 2007; IRL Press at Oxford University Press, Oxford.

18 Balows A, Hausler WJ, Herrmann KL, Isenberg HD and Shadomy HJ: Manual of Clinical Microbiology. 2007 ASM Press, Washington, D.C.

19 Palmer AGI, Cavanagh J, Wright PE, Rance M *et al*: Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectroscopy. J Magn Resonance *93*: 115-170, 1991.

20 Schleucher J, Schwendinger M, Sattler M, Schmidt P, Schedletzky O, Glaser SJ, Sørensen OW and Griesinger C: A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. J Biomol NMR *4*: 301-306, 1994.

21 Cavanagh J, Fairbrother WJ, Palmer AGI and Skelton NJ: Protein NMR Spectroscopy – Principles and Practice Second Edition. 2007; Elsevier Academic Press, U.S.A.

22 Butt HL, Dunstan RH, McGregor NR, Roberts TK, Zerbes M and Klineberg IJ: Alteration of the bacterial microbial flora in chronic fatigue/pain patients. The clinical and scientific basis of chronic fatigue syndrome: From myth towards management, Manly Australia, 1998.

23 Evengard B, Nord CE and Sullivan A: Patients with chronic fatigue syndrome have higher numbers of anaerobic bacteria in the intestine compared to healthy subjects. Eur Soc Clin Microbiol Infect Dis *17*: S340, 2007.

24 Stolberg L, Rolfe R, Gitlin N, Merritt J, Mann L Jr, Linder J and Finegold S: D-Lactic Acidosis due to Abnormal Gut Flora. N Engl J Med *306*: 1344-1348,1982.

25 Savage DC: Microbial ecology of the Gastrointestinal tract. Annual Rev Microbiol *31*: 107-133, 1977.

26 Hoogkamp-Korstanje JA, Linder J, Marcelis JH, den Daas-Slagt H and de Vos NM: Composition and ecology of the human intestinal flora. Antonie van Leeuwenhoek *45*: 35-40, 1979.

27 Ducluzeau R: Role of experimental microbial ecology in gastroenterology. *In*: Microbial Ecology and Intestinal Infections. Bergogne-Berezine E (ed.). Springer, Paris, 1989.

28 Smith HW and Crabb WE: The faecal bacterial flora of animals and man: its development in the young. J Pathol Bacteriol *82*: 53-66, 1961.

29 Noble CJ: Carriage of group d streptococci in the human bowel. J Clin Pathol *31*: 1182-1186, 1978.

30 Benno Y, Endo K, Mizutani T, Namba Y, Komori T and Mitsuoka T: Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. Appl Environ Microbiol *55*: 1100-1105, 1989.

31 Caldarini MI, Pons S, D'Agostino D, DePaula JA, Greco G, Negri G, Ascione A, Bustos D. Fecal flora in a patient with short bowel syndrome. An *in vitro* study on effect of pH on D-lactic acid production. Dig Dis Sci *41*: 1649-1652, 1996.

32 Kamar M, Raziel A, Susmallian S, Kyzer S and Charuzi I: D-Lactic Acidosis in a Patient after Subtotal Coloectomy. Isr Med Assoc J *5*: 891-892, 2003.

33 Hutkins RW and Nannen NL: pH homeostasis in lactic acid bacteria. J Dairy Science *76*: 2354-2365, 2008.

34 Henriksson AEK, Tagesson C, Uribe A, Uvnäs-Moberg K, Nord CE, Gullberg R and Johansson C: Effects of prostaglandin E2 on disease activity, gastric secretion and intestinal permeability, and morphology in patients with rheumatoid arthritis. Ann Rheum Dis *47*: 620-627, 1988.

35 Zhang DL, Jiang ZW, Jiang J, Cao B and Li JS: D-lactic acidosis secondary to short bowel syndrome. Postgrad Med J *79*: 110-115, 2003.

36 Inoue Y, Shinka T, Ohse M, Kohno M, Konuma K, Ikawa H and Kuhara T: Changes in urinary level and configuration ratio of D-lactic acid in patients with short bowel syndrome. J Chromatogr B Analyt Technol Biomed Life Sci *855*: 109-14, 2007.

37 Maes M, Mihaylova I and Leunis JC: Increased serum IgA and IgM against LPS of enterobacteria in chronic fatigue syndrome (CFS): indication for the involvement of gram-negative enterobacteria in the etiology of CFS and for the presence of an increased gut-intestinal permeability. J Affect Dis *99*: 237-240, 2007.

38 Chia JKS: The role of enterovirus in chronic fatigue syndrome. J Clin Pathol *58*: 1126-1132, 2005.

39 Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, Kazzaz Z, Bornstein E, Lambotte O, Altmann D, Blazar BR, Rodriguez B, Teixeira-Johnson L, Landay A, Martin JN, Hecht FM, Picker LJ, Lederman MM, Deeks SG and Douek DC: Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med *12*: 1365-1371, 2006.

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