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Original Research Article

Elimination of high-refined-sugar diet as treatment strategy for autistic features induced in a rodent model

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Abstract

Purpose: *To investigate the potency of ampicillin in altering gut flora in the presence of a high-sucrose diet in rat pups, and to determine its effect on selected neurotransmitters and a cytokine as markers of the persistent autistic features repeatedly induced in orally administered propionic acid rat pups..*
Methods: *Twenty-eight young male Wistar albino rats were divided into four equal groups. The first group served as a control. The second group received an oral neurotoxic dose of propionic acid (PPA, 250 mg/kg body weight/day) for 3 days. The third group was treated with ampicillin (50 mg/kg for 3 weeks) with a standard diet. The fourth group was given the same dose of ampicillin with a high-sucrose diet for 10 weeks.*
Results: *The results showed a significant ($p < 0.001$) decrease in the investigated neurotransmitters in PPA- and ampicillin-treated rat pups (norepinephrine by 32.49 and 14.58 %, dopamine by 31.45 and 20.22 %, serotonin by 35.99 and 29.09 %), as well as a remarkable increase ($p < 0.001$) in the pro-inflammatory cytokine, IL-6 (30.07and 6.07 %). The high-sucrose diet also significantly ($p < 0.001$) enhanced the neurotoxic effect of ampicillin.*
Conclusion: *The observed dietary modulation of the gut microbiota, coupled with the subsequent modulation of brain neurochemistry and inflammation, demonstrates the considerable potential of dietary intervention through the elimination of highly refined sugar as a treatment strategy to prevent and treat autism.*

Keywords: *Neurotoxicity, Ampicillin, Propionic acid, Neurotransmitters, Cytokines, High-sucrose diet*

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INTRODUCTION

Antibiotics are prescribed frequently for infants and children in nearly all pediatric clinics [1].Antibiotic treatment over a long period results in a 10-fold increase of Clostridium spp (propionibacteria) in the gut. Overgrowth of this species has also been reported in autistic patients [2].Furthermore, the induction of persistent autistic features in rodent modelswas achieved by MacFabe *et al* [3] and El-Ansary *et al* [4] through propionic acid (PPA) exposure. A concomitant increase in the fecal concentrations of PPA in children with autism may indicate the role by which microbial metabolites alter brain function through the gut–brain axis. It is now acknowledged that the gut–brain axis and balanced gut microbiota are critically important during brain development and can play a major role in the pathogenesis of autism. In the gut, several members of the *Bifidobacterium* and *Lactobacillus* genera have been found to

possess health-promoting properties and are affected by diet, whereas many *Proteobacteria* have the potential to become pathogenic when a suitable environment is provided. Due to immature renal function and blood brain barrier, ampicillin is neurotoxic in very low birth weight neonates, resulting in a remarkable increase of ampicillin in both serum and CSF [5]. Ampicillin is believed to exert an inhibitory effect on GABA transmission [6]. Imbalances in GABAergic/glutamatergic, serotonergic, and dopaminergic neurotransmission together with neuroinflammation were recently reported as the most important signals related to the clinical presentation and severity of autism [7].

This information motivated our interest to compare the gut–brain neurotoxic effect of ampicillin to that induced by orally administered PPA as a metabolic end-product of propionibacteria. The impact of a high-refined-sugar diet on the neurochemical effects induced by ampicillin was also assessed.

EXPERIMENTAL

Animals

The study included 28 male Wistar albino rats divided into four independent groups containing seven rats each. Group I served as the control and received only phosphate buffered saline; Group II was administered with PPA (250 mg/kg body weight/day) for three days [4]; Group III was treated with an oral dose of ampicillin (50 mg/kg for three weeks) with a standard diet; and Group IV was given the same dose of ampicillin with a high-sucrose diet for 10 weeks. The study was approved by the Ethics Committee of King Saud University with approval no.KSU-IRB008E. International animal care guidelines were strictly followed [8].

Diet

The special diet characterized by high sugar was made by mixing sucrose with regular chow.

Sample preparation

Brain tissue was homogenized in 10 times w/v bi-distilled water and was used to measure all parameters evaluated in the study.

Assay for neurotransmitters (NA, DA and 5HT)

High-performance liquid chromatography with electrochemical detection was used to measure dopamine, serotonin and norepinephrine [9].

IL-6 assay

IL-6 was assayed using a Quantikine ELISA kit (R & D Systems, Minneapolis, MN, USA). A microplate was pre-coated with a monoclonal antibody specific for rat IL-6.

Glutamine and glutamate assays

Rat brain glutamine and glutamate were measured independently using Cusabio ELISA Kits.

Microbiological examination

Fecal samples were used to identify the gut bacteria by culturing the samples under aerobic and anaerobic conditions. All the species found were identified using Scepter micro dilution and standard bacteriological techniques.

Statistical analysis

SPSS (Chicago,IL, USA) was used to analyze the data by using ANOVA for one-way analysis of variance and Dunnett test for multiple comparisons. P values less than 0.05 were considered significant. Receiver operating characteristics curve (ROC) analysis was performed to test the predictive value of the investigated parameters and to measure the toxicity of PPA and ampicillin in intoxicated rats. An area under the curve (AUC) of 0.8-1 indicated an excellent predictive value for the biomarker. This was accompanied by satisfactory values for sensitivity and specificity. Pearson's correlation analysis was also performed using the same SPSS program. Multiple regression analysis was conducted using glutamate and IL-6 as two dependent variables against the rest of the variables, which were used as independent or predictor variables.

RESULTS

Ampicillin treatment for six weeks altered the gut flora, resulting in unusual growth of a few PPA producers (e.g., *Klebsiella pneumonia* and *Proteus vulgaris*), while some species, such as *Enterobacter cloacae*, vanished. Ampicillin also promoted the overgrowth of *Candida albicans.*

Table 1 and Figure 1 show the significant changes in the brain chemistry of rats treated with PPA and ampicillin, as well as changes in those fed a high sucrose diet for 10 weeks post antibiotic treatment. NA was significantly reduced in both PPA and ampicillin-treated groups by 32.49 % and 14.58 %, respectively. However, the high-sucrose diet in ampicillin-treated rats was

the most potent, as it induced a 43.11 % decrease in NA in the brain. The three groups differed from the control ($p < 0.001$). However, PPA induced the most significant decrease in DA and 5HT.

Regarding the excitotoxicity presented by glutamate, PPA was the most potent, as it induced a 56.3 % increase, followed by the other two groups, which demonstrated a 23.55 and 17.75 % increase, respectively. The glutamine level was equally affected in the different treatments, showing an approximately 35 % increase *at* $p < 0.013$. The glutamate/glutamine ratio showed a non-significant difference compared to the control (Figure 1 and Table 2).

IL-6 was remarkably elevated in all experimental groups compared to that of the control. PPA and a HSD induced similar increases in IL-6 (30 % and 27 %, respectively), which were noticeably higher than that in ampicillin-treated animals (6.07 %) (Table 1 and Figure 1).

Table 1: Biochemical parameters of PPA, ampicillin-treated and ampicillin/HSD-fed rats

Parameter	Group	N	Mean ± S.D.	Percent change	*P*-value
NA	Control	7	6.92 ± 0.78	100.00	0.001
	PPA	7	4.67 ± 0.45	67.51	
	Ampicillin	7	5.91 ± 0.28	85.42	
	HSD	7	3.93 ± 0.45	56.89	
DA	Control	7	25.95 ± 1.68	100.00	0.001
	PPA	7	17.79 ± 1.34	68.55	
	Ampicillin	7	20.70 ± 3.37	79.78	
	HSD	7	21.46 ± 0.78	82.72	
IL-6	Control	7	227.34 ± 19.47	100.00	0.001
	PPA	7	295.71 ± 23.92	130.07	
	Ampicillin	7	241.15 ± 23.26	106.07	
	HSD	7	290.22 ± 41.24	127.66	
5-HT	Control	7	8.59 ± 0.42	100.00	0.001
	PPA	7	5.50 ± 0.66	64.01	
	Ampicillin	7	6.02 ± 0.51	70.10	
	HSD	7	6.03 ± 0.55	70.25	
Glut	Control	7	237.99 ± 18.93	100.00	0.007
	PPA	7	371.97 ± 105.29	156.30	
	Ampicillin	7	294.03 ± 57.76	123.55	
	HSD	7	280.22 ± 47.12	117.75	
Gln	Control	7	2169.73 ± 223.94	100.00	0.013
	PPA	7	2930.67 ± 664.56	135.07	
	Ampicillin	7	2921.69 ± 520.35	134.66	
	HSD	7	2967.29 ± 412.29	136.76	
Glut/ Gln	Control	7	9.15 ± 1.10	100.00	0.183
	PPA	7	8.36 ± 2.80	91.35	
	Ampicillin	7	10.15 ± 2.02	110.89	
	HSD	7	10.82 ± 2.37	118.24	

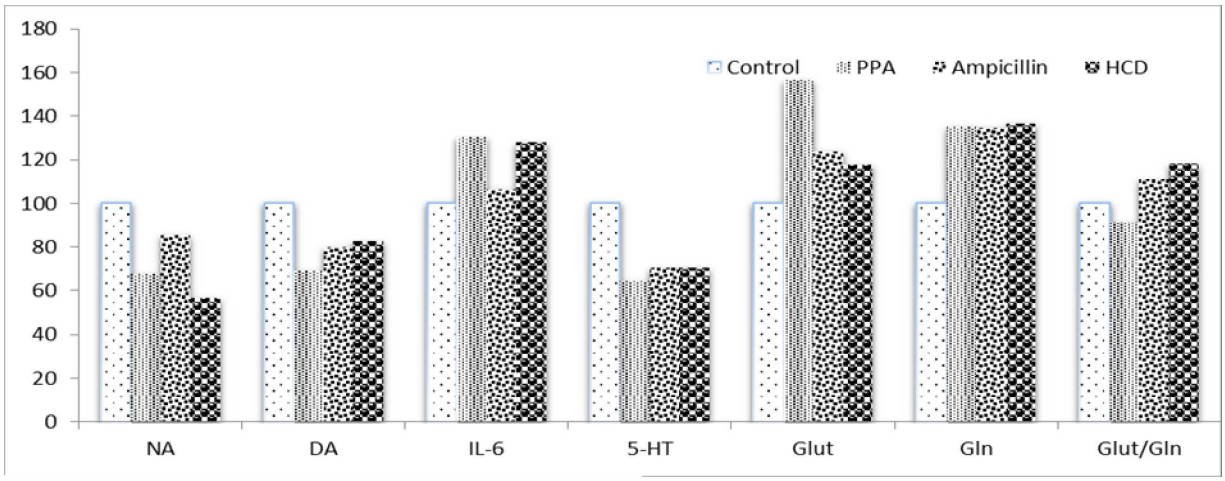

Figure 1: Percent change in all measured parameters

Table 2: Pearson's correlation between the measured parameters

Parameter	R (Pearson's correlation)	P value
NA ~ DA	0.496	0.007
NA ~ IL-6	-0.645	0.001
NA ~ 5-HT	0.678	0.001
NA ~ Gln	-0.556	0.002
DA ~ IL-6	-0.563	0.002
DA ~ 5-HT	0.756	0.001
DA ~ Glut	-0.508	0.006
DA ~ Gln	-0.509	0.006
IL-6 ~ 5-HT	-0.610	0.001
IL-6 ~ Gln	0.563	0.002
IL-6 ~ Glut/Gln	0.374	0.050
5-HT ~ Glut	-0.466	0.012
5-HT ~ Gln	-0.639	0.001
Glut ~ Glut/Gln	-0.592	0.001
Gln ~ Glut/Gln	0.545	0.003

Table 2 presents the Pearson's correlations between the measured parameters. It is clear that the neuroinflammatory marker IL-6 was positively correlated with glutamine and glutamate/glutamine ratio and was negatively correlated with NA, DA and 5HT. In addition, NA, DA and 5HT were positively correlated with each other and negatively correlated with glutamate, glutamine and glutamate/glutamine ratio.

Table 3 and Figure 2 present the area under the curve (AUC), specificity and sensitivity for the measured neurotransmitters and for IL-6 in the studied groups.

Table 4 shows the results of multiple regression analysis using glutamate as a dependent variable. It can be easily confirmed that glutamate is related to glutamine and the glutamate/glutamine ratio, recording R^2 values of 0.896.

DISCUSSION

As gastrointestinal conditions and eating problems are common in autism, diet might represent a link between environmental and neurobiological factors and thus may play a role in the pathways leading to the etiology of this disorder. The impact of PPA, ampicillin treatment and a high-sucrose diet on the alteration of brain chemistry will be discussed. The recorded overgrowth of *Candida albicans* in the gut can be easily related to the induced autistic features. Yeast feed on sugar and produce alcohol, a toxic chemical that can easily be transported in the blood to the frontal lobes of the brain, where it

Table 3: ROC analysis of the measured parameters demonstrating area under the curve (AUC), cutoff values, sensitivity and specificity in the three experimental groups.

Variable	Group	Area under the curve	Cutoff value	Sensitivity (%)	Specificity (%)
	PPA	1.000	5.420	100.0	100.0
NA	Ampicillin	0.918	6.365	100.0	85.7
	HCD	1.000	5.220	100.0	100.0
	PPA	1.000	21.950	100.0	100.0
DA	Ampicillin	0.918	23.675	85.7	100.0
	HCD	1.000	23.435	100.0	100.0
	PPA	1.000	258.510	100.0	100.0
IL-6	Ampicillin	0.673	252.070	42.9	100.0
	HCD	0.959	252.260	85.7	100.0
	PPA	1.000	7.115	100.0	100.0
5-HT	Ampicillin	1.000	7.300	100.0	100.0
	HCD	1.000	7.380	100.0	100.0
	PPA	1.000	267.650	100.0	100.0
Glut	Ampicillin	0.878	257.650	85.7	85.7
	HCD	0.816	258.140	71.4	85.7
	PPA	0.918	2551.090	71.4	100.0
Gln	Ampicillin	0.959	2517.300	85.7	100.0
	HCD	1.000	2514.155	100.0	100.0
	PPA	0.612	7.436	42.9	100.0
Glut/Gln	Ampicillin	0.694	9.185	71.4	71.4
	HCD	0.755	9.385	85.7	71.4

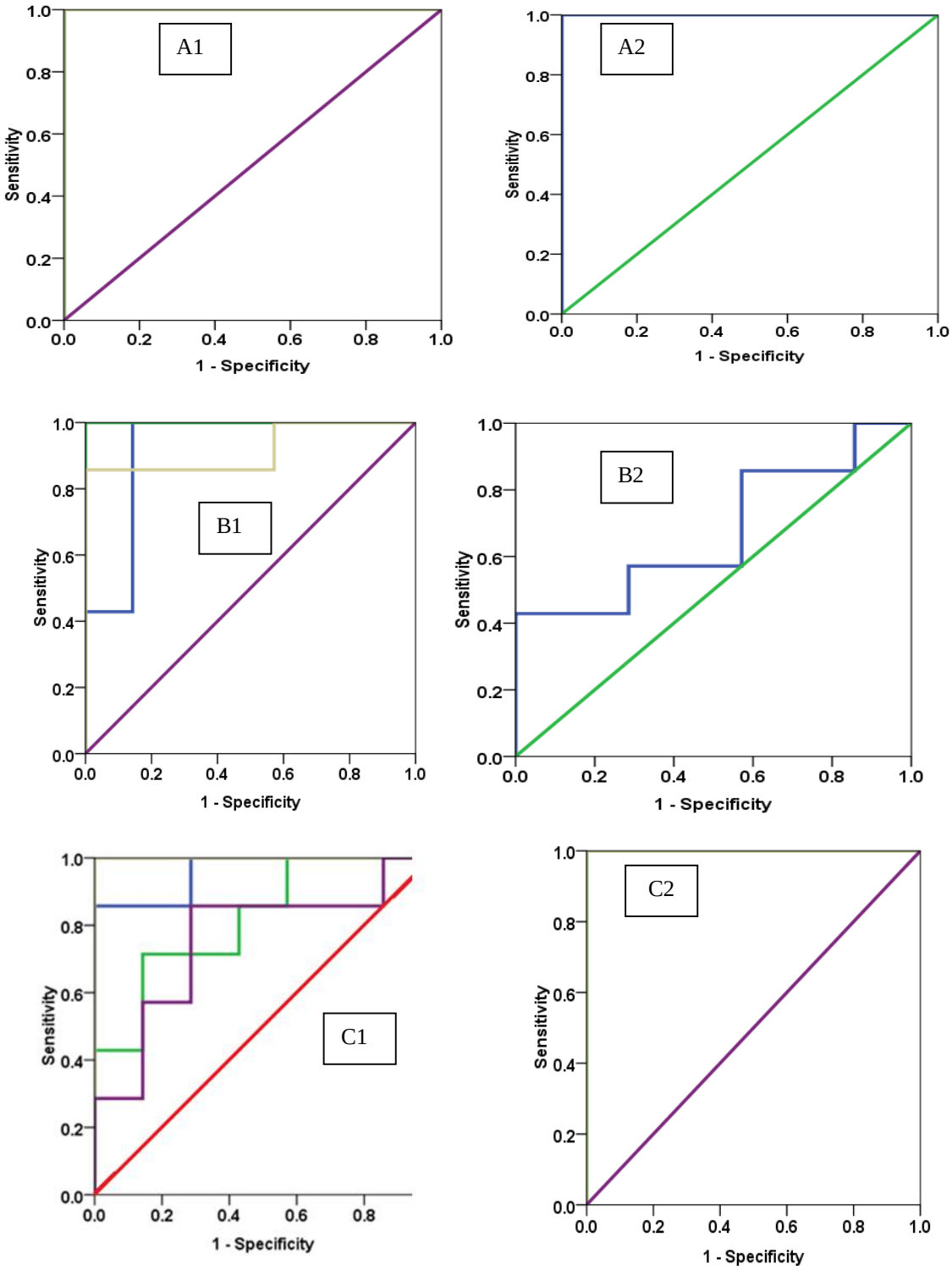

Figure 2: ROC Curve for all parameters in propinic acid group (A), ampicillin group (B) and ampicillin-HSD fed group (C)

Table 4: Multiple regression using stepwise method for glutamate as a dependent variable

Predictor variable	Beta	*P*-value	Adjusted R square	Model F-*value*	Model *P-value*
Glut/Gln	-1.075	0.001	0.896	117.714	0.001
Gln	0.887	0.001			

inhibits nerve growth and brain development. This inhibition often manifests as sugar cravings, hyperactivity, irritability, social impairment and cognitive disability [10].

Table 2 and Figure 1 show that PPA, ampicillin and a high-refined-sugar (sucrose) diet induce noticeable alterations in brain chemistry that manifest as a significant decrease or increase in almost all the measured neurotransmitters. This is related to the gut microbiota changes induced either by PPA, ampicillin or highly refined sugar during ampicillin treatment. This theory is supported by the recent study by Heijtz *et al* [11], who confirmed that after gut microbiota are disrupted by multiple factors including antibiotics, CNS chemistry is badly affected through the gut–brain axis.

Imbalances in the composition of gut microbiota in PPA or ampicillin-treated rats are observed as the overgrowth of *K. pneumonia and P. vulgaris,* which are propionibacteria and inducers of propionic acid producers, respectively. The concomitant alteration of most of the measured neurotransmitters together with the immune sequelae presented by the increase of IL-6, as shown in Table 2, may contribute to the development of persistent autistic features in treated rats [12].

The significant decrease in the concentration of NA in the three groups investigated (PPA-treated, ampicillin-treated and ampicillin-HSD fed) demonstrates the neurotoxic effects of PPA, ampicillin and HSD, as this neurotransmitter is essential for a wide range of CNS tasks and is also involved in neuronal growth [13]. NA has also been shown to limit neuroinflammation in the CNS [14]. This result can also be easily related to the significant elevation of IL-6, TNF α, and IFN γ in the brain homogenates of PPA-treated rats [4]. This could provide support for the contribution of PPA and ampicillin as environmental factors related to altered gut-microbiota in the pathogenesis of autism.

The remarkable decrease in 5-HT in the three groups studied can be easily related to the imbalance between inhibitory/excitatory transmission reported in autistic patients and rodent models of autism [4,15]. 5-HT interacts with DA, NE, GABA, and glutamate systems [16,17] and is critical for the maturation of the GABA phenotype in the ventral spinal cord via 5-HT1B receptors [16]. Furthermore, an interaction between the glutamate receptors mGLU5 and the 5-HT2A receptor was shown in mice [17]. The remarkable decrease of serotonin level in the brain of treated rats can be related to the toxic effects of PPA [3] or the association between microbiota and serotonin signaling. Both the ampicillin-treated and ampicillin-HSD fed groups demonstrated altered intestinal microbiota composition, which can easily relate to the remarkable decrease in serotonin [18].

Table 1 and Figure 1also demonstrate the significant decrease of the neurotransmitter DA in the three groups studied. This decrease can be attributed to the effects of PPA, ampicillin and ampicillin-HSD on DA transporters, thus increasing DA breakdown. This suggestion can find support in the work of Bello *et al* [19], who demonstrated that 20 min of access to 0.3 M sucrose for only 6 days results in upregulation of the DA membrane transporters and downregulation of the DA receptors.

The significant increase in brain glutamate upon treatment with PPA can be easily related to ASD features. Glutamate excitotoxicity is one of the most important mechanisms involved in the etiology of autism. The elevation of glutamate, the main brain excitatory neurotransmitter, in the PPA, ampicillin-treated or ampicillin-HSD groups demonstrated excitotoxicity in the three groups studied. Elevation of glutamate can find support in a previous study [20], which reported that decreased GABA, an inhibitory neurotransmitter, could alter excitation/inhibition balance and lead to less inhibition of glutamatergic inputs in PPA-treated rat pups.

On the other hand, the significant increase of glutamate as a marker of excitotoxicity in ampicillin–treated animals contradicts a previous report by Rothstein *et al.* [21]. The report declared that several β-lactam antibiotics, to which ampicillin belongs, upregulate the expression of the glutamate transporter GLT-1 protein on astrocytes, thus reducing synaptic glutamate and contributing to decreased glutamate excitotoxicity. This inconsistency can be attributed to the substantial variation in the doses used. In the present study, 50 mg/kg body weight was used with the intention to alter gut microbiota and to study the effect of the induced overgrowth of PPA-related bacteria. The persistent elevation of glutamate in ampicillin-treated rats fed a high-sucrose diet can be supported by the recent report by Kuang *et al* [22]. The study demonstrated that a maternal high-sucrose (HS) diet affects behavior and hippocampal neurons in the young offspring, leading to learning deficits through a significant increase in the expression of N-methyl-D-aspartate (NMDA) receptors. These receptors serve as modulators of glutamate synaptic transmission, and thus excessive activation can

reflect glutamate elevation and the disruption of calcium homeostasis, resulting in either apoptotic or necrotic neuronal death or both [23].

The significant increase of glutamine reported in the present study with the concomitant decrease of glutamate/glutamine ratio in PPA-treated rats can be supported by the recent study by Davison *et al* [24]. The authors performed neuroimaging using magnetic resonance spectroscopy (MRS) and demonstrated glutamine/glutamate elevation in the basal ganglia of all propionic acidemia patients during stable metabolic conditions. They attributed this elevation to the impact of hyperammonemia on cerebral brain water due to glutamine being osmotically active. The elevation of glutamine in ampicillin-treated or ampicillin-HSD animals can be related to the altered microbiota, as presented in Table 1. Growth was detected in *K. pneumonia,* opportunistic pathogens known to generate ammonia in immune-compromised humans and animals, and hyperammonemia, which usually contributes to the pathogenesis of this bacterial species and clostridia as propionibacteria (not detected with the microbiological technique used in the present study) [25].

Table 3 shows the remarkable association between the impaired neurotransmitters and 1L-6. This association demonstrates that both neurochemistry alteration and neuroinflammation are involved in the neurotoxic effects of PPA and ampicillin. The results also confirms the toxic effect of HSD during antibiotic treatment.

The results demonstrate the satisfactory AUC, specificity and sensitivity of all measured parameters as markers for PPA, ampicillin and HSD.

CONCLUSION

The use of ampicillin influences the profile of microbiota through gut–brain interactions that affect brain neurochemistry and displays the risk of inducing biochemical autistic features. The remarkable effects of a high-sucrose diet on most of the measured parameters show that the induction of PPA-related microbial growth interacts with diet as an environmental risk factor.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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