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Pilot study of the SCFA Headspace Analysis of *Streptococcus mutans* Metabolites in Media with and without Polyols

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Abstract

This pilot study of *Streptococcus mutans* ATCC 35668 grown in media with and without polyols (erythritol) measured the resultant metabolites, including the Short Chain Fatty Acids (SCFA) by using head space analysis. Brain Heart Infusion Broth (BHI2 or BHI10) supplemented with 2% or 10% sucrose containing no polyols or either erythritol or xylitol and *Streptococcus mutans* (ATCC 35668) was grown aerobically. After 48 hours of growth the supernatant were harvested and centrifuged to pellet bacteria. Supernatants were removed from bacterial pellets then submitted for SCFA analysis with an Agilent Technologies (Santa Clara, CA 95051) system configured from three components, a 5973-mass selective detector, a 6890N gas chromatographer, and a 7697A headspace sampler. *Streptococcus mutans* growing in BHI supplemented with 2% or 10% sucrose but containing no polyols produced the following short chain fatty acids: methyl isovalerate, acetic acid, propionic acid, butanoic acid, pentanoic acid, ethyl butaric acid, 4-methylvaleric acid, hexanoic acid. When the BHI broth supplemented with 2% or 10% sucrose containing erythritol was used as media for this *Streptococcus mutans* strain, the following were produced: ethanol, acetoin, and acetic acid. Our results would indicate that constituents of the bacteria media may affect the bacterial metabolite production.

Keywords: *Streptococcus mutans*, Polyols, Gut microbiome.

Introduction

Very little research has actually been focused on the short chain fatty acid SCFA production of one of mankind's most prevalent pathogens, *Streptococcus mutans*. *S. mutans* is a key dental pathogen, long associated with one of the most common diseases of humankind [1]. The incidence of dental caries in over 98% of the 65 years and above population demonstrates the universality of this disease [2]. However, dental caries is totally preventable, being the result of a dysbiosis of the oral cavity, with both the increased presence of oral pathogens and the decreased level of protective commensals, particularly the nitrate reducing commensals [3]. The oral microbiome shifts significantly over the different time periods of child development and in response to the diet [4]. Unfortunately, the oral microbiome has had the same response as the Gut microbiome to the massive dietary shifts; the Agricultural, Industrial, and more currently, the Fast Food revolutions [5]. That is, there has been a relative decrease in diversity coinciding with an increase not only in the number of pathogens, but also their pathogenicity [6].

Efforts to reduce the levels of *S. mutans* in infants and children with xylitol and preventing dental caries have been successful [7,8]. However, other bacterial and fungal organisms have now been closely identified with the development of dental caries [9]. *Scardovia wiggsiae* is a Bacillus bacteria found extensively associated with Severe-Early Childhood Caries (S-ECC) [10]. *Scardovia wiggsiae* and *Slackia exigua* have been reported to be involved in the early caries development [11]. *Candida albicans*, a fungal organism, helps with the biofilm production by increasing the extracellular polysaccharide matrix which protects *S. mutans* from anti-microbials and commensals such as *Streptococcus oralis* [12]. Lactobacilli inhibit the colonization of *Candida albicans*, hence decreasing the polysaccharide matrix, exposing the *S. mutans* to the bactericins or hydrogen peroxide of its natural competitors, other Streptococcus species [13]. In addition, *Streptococcus oralis* produces hydrogen peroxide that inhibits the anaerobic *Streptococcus mutans* growth [14,15]. Indeed, Probiora© probiotic, a commercially available probiotic product, contains *Streptococcus oralis, uberis and rattus*, and claims to inhibit several key dental pathogens [16-19]. Probiotics have been reported to be an important adjunct in preventive dental care [20-22]. Xylitol has been studied for its effect on the lactobacillus bacteria, a genus that consists of many probiotics, and it has been reported that xylitol does not significantly inhibit the Lactobacilli.

Polyols, sugar alcohols, have a distinct effect upon the microbiome and have long been utilized in oral medicine to reduce pathogen populations and also are referred to as prebiotics. Significant research studies have long demonstrated the effectiveness of polyol ingestion for the prevention of dental caries and now also for periodontal pathology [23,24]. A significant portion of the effectiveness is reportedly due to the polyol effect on the pathogenic microbiome [25].

Pathogens are more susceptible to the inhibitory effect of xylitol than the commensal bacteria. Studies of xylitol demonstrated little effect on probiotic bacteria, and long clinical studies demonstrate the biofilm effects are long term, and even are transmissible from mother to child [26,27]. Polyols safely inhibit the growth and biofilm production of oral pathogens that also have a significant effect systemically, such as, *S. mutans* causing hemorrhagic stroke [28]. In addition, polyols shift the metabolites (acetate, lactate and propionate) produced by the oral microbiome [29]. Carious dentin contains both acetate and propionate, produced by cariogenic bacteria prompting the research into the propionic acid production by *S. mutans* [30]. Polyols have been reported to shift the production of the organic acids of the oral microbiome in the young patient population creating a long term benefit [31].

Materials and Methods

BHI broth supplemented with 2% or 10% sucrose containing no polyols or either erythritol or xylitol at various concentrations was used for this study. *S. mutans* (ATCC 35668) was grown aerobically. After 48 hours of growth the supernatant were harvested and centrifuged to pellet bacteria. Supernatants were removed from bacterial pellets, filtered through 0.22 micron filters and stored in sterile cryovials until submitted for SCFA analysis at the IMSERC Mass Spectrometry Center (Northwestern University).

The instrument utilized was an Agilent Technologies (Santa Clara, CA 95051) system configured from three components, a 5973 mass selective detector, a 6890N gas chromatographer, and a 7697A headspace sampler. Mixture components separation was achieved by using a FFAP column (Agilent J&W DB-FFAP; is a nitroterephthalic-acid-modified PEG) and a 10 minute temperature gradient (initial temperature at 50 °C, hold for 1 minute, and ramp to 240 °C in 6 minutes, and held for 3 minutes, to give a total run time of 10 minutes). The standards of each of the SCFA samples were made in water and linearity established before test samples were committed to analysis. The linearity of the test samples were also demonstrated before the data was accepted. The SCFA test samples were analyzed as submitted without need for any further processing. Headspace oven incubation times of 15 minutes were used for both test samples and standard solutions.

Results

Representative Data

Standard Positive Control Negative Control

	Acetic Acid (m/z 60 Area cnts)	Sample Volume (µL)	On column (ng/µL)
Basal Media Neg Control	137694	10	1.05
CX-Sup-48hrs-Pos-Cont	1786539	10	13.60

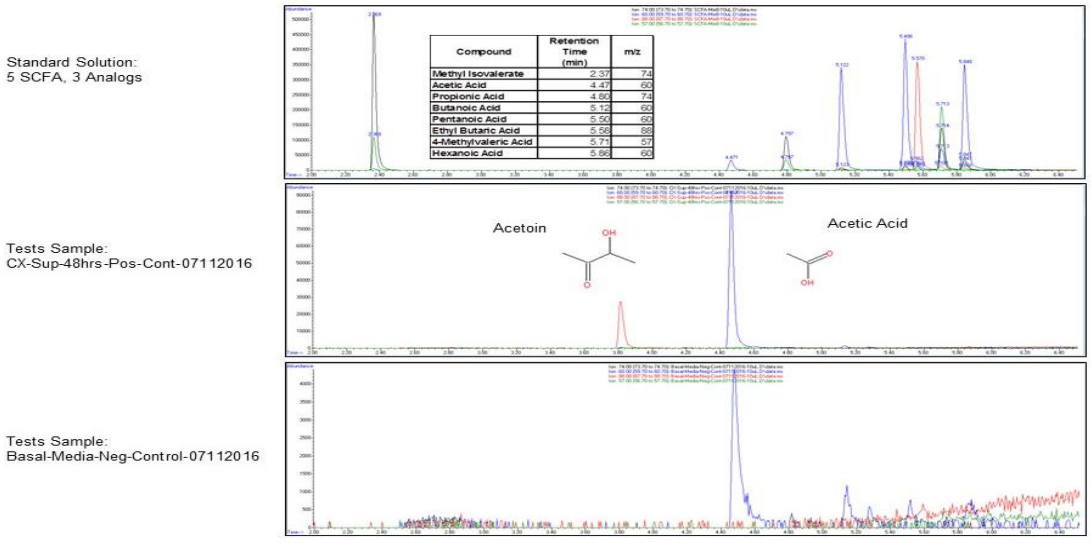

Figure 1: Headspace-GCMS representative chromatograms (a) Standard mix containing five short chain fatty acids and three analogs. Inset table shows retention time and m/z of each component. (b) Positive control sample prepared by using CX-SUP-48hrs. (c) Negative control sample prepared by using Basal Media.

When the BHI broth was supplemented with 2% or 10% sucrose but containing no polyols was used to grow *S. mutans*, the following short chain fatty acids were produced: methyl isovalerate, acetic acid, propionic acid, butanoic acid, pentanoic acid, ethyl butaric acid, 4-methylvaleric acid, hexanoic acid. Note that this particular strain of *S. mutans* did not produce lactic acid. When the BHI broth supplemented with 2% or 10% sucrose containing erythritol was used as media for this *S. mutans* strain, the following were produced: ethanol, acetoin, and acetic acid. Note that propionic acid was not detected.

Compound	Retention Time (min)	m/z
Methyl Isovalerate	2.4	74
Acetic Acid	4.5	60
Propionic Acid	4.8	74
Butanoic Acid	5.1	60
Pentanoic Acid	5.5	60
Ethyl Butaric Acid	5.6	88
4-Methylvaleric Acid	5.7	57
Hexanoic Acid	5.9	60
Ethanol	2.0	45
Acetoin	3.8	45

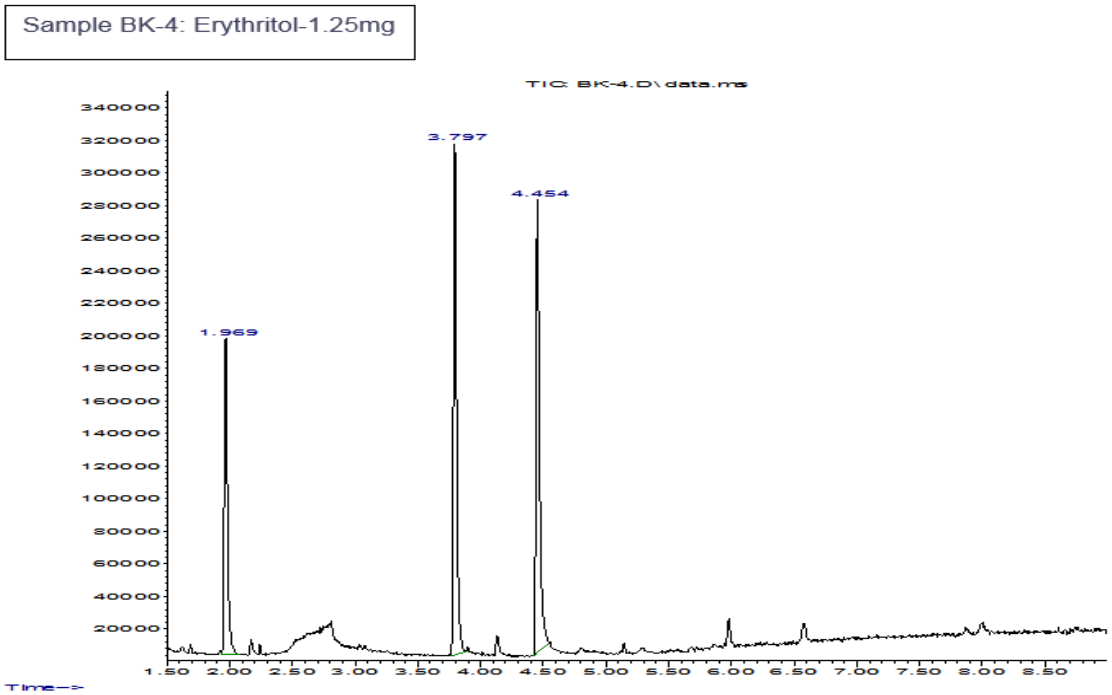

Figure 2: BK-4 test sample headspace-GCMS total ion chromatogram showing the presence of ethanol at retention time 2.0 minutes, Acetoin at retention time 3.8 minutes, and Acetic Acid at retention time 4.5 minutes. (Retention time and m/z table is provided as a guide).

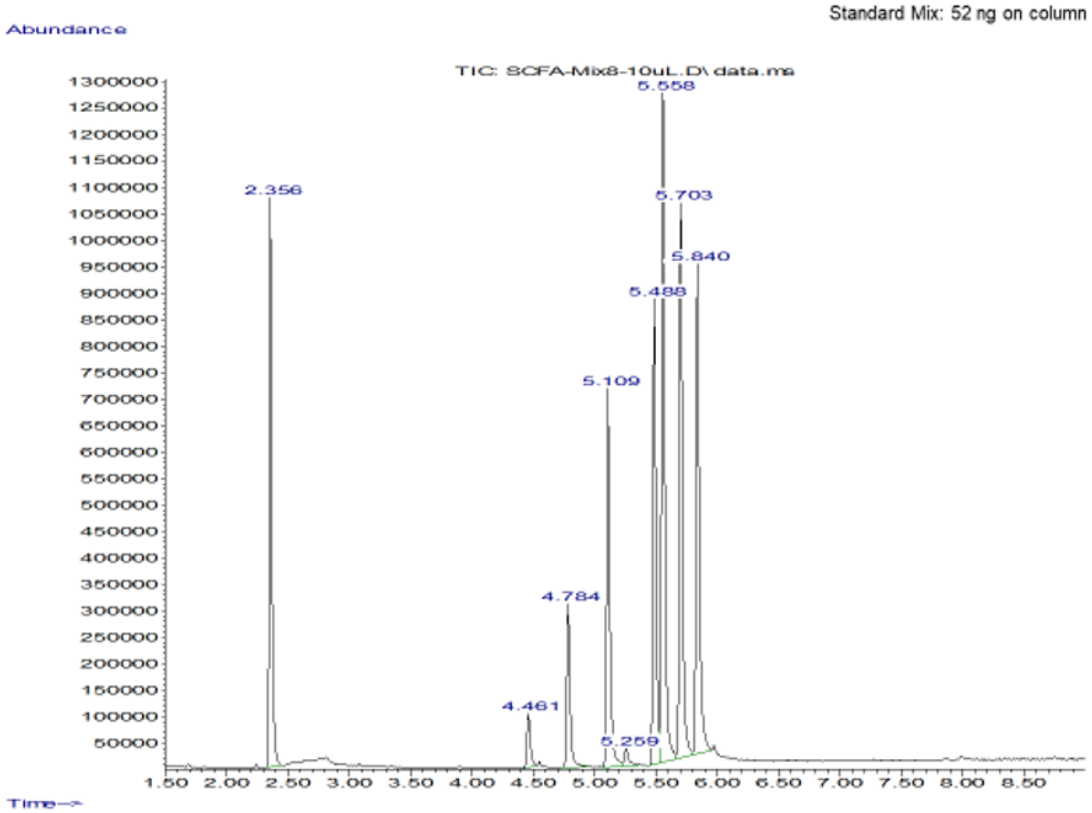

Figure 3: Headspace-GCMS total ion chromatogram of standard mix (5 SCFA and 3 analogs) from a 52ng injection of each component on column. Inset table provided as a guide showing the retention times and identifying m/z value for each component.

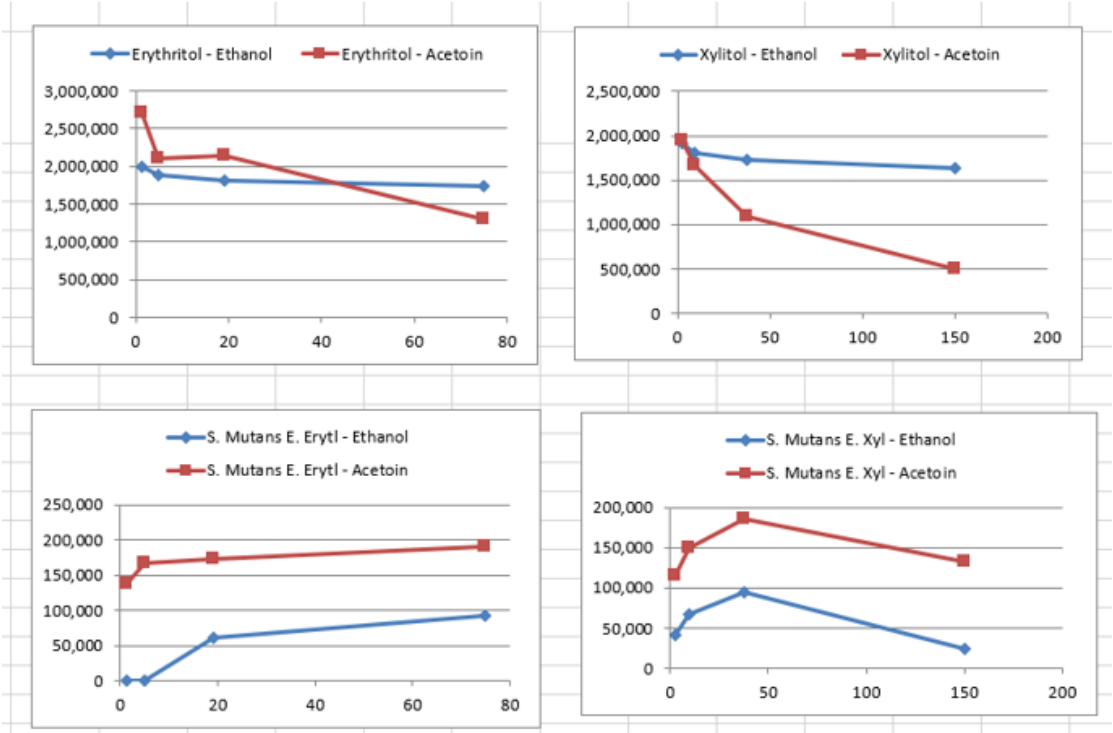

Figure 4: Evidence of modification of microbiome metabolites with polyols. Graphs showing signal intensities for ethanol (a) and acetoin (b) trends relative to the amount of Erythritol and Xylitol (x axis, in mg) added to the growth media without the presence of *S. mutans*. In the presence of *S. mutans* and Erythritol (c) and Xylitol (d), ethanol and acetoin signal intensities are significantly affected.

							No Quantitation; Area counts only	
Sample Number	**File Name**	**Sample ID**		**Acetic Acid (m/z 60 Area cnts)**	**Sample Volume (uL)**	**Conc. (ng/uL)**	**Ethanol (Ret. Time 1.97 min, m/z 45)**	**Acetoin (Ret. Time 3.80min, m/z 45)**
0	**Blank-1**	**Air Blank**		**0**	**0**	**0**	**0**	**0**
1	BK-1	+ control, 10% sucrose		117677	10	0.87	**0**	**0**
2	BK-2	S. Mutans control (-) media		103519	10	0.76	**0**	**0**
3	BK-3	S. Mutans E. control (+) Opolyol		134802	10	1.00	**0**	**0**
0	**Blank-2**	**Air Blank**		**0**	**0**	**0**	**0**	**0**
4	BK-4	Erythritol-1.25mg	1.25	1608577	10	11.88	2001284	2703626
5	BK-5	Erythritol-5mg	5	1623546	10	11.99	1880777	2097052
6	BK-6	Erythritol-19mg	19	1544335	10	11.40	1804859	2131294
7	BK-7	Erythritol-75mg	75	1434725	10	10.59	1734627	1291860
0	**Blank-3**	**Air Blank**		**0**	**0**	**0**	**0**	**0**
8	BK-8	Xylitol-2.3mg	2.3	1716499	10	12.67	1914897	1949298
9	BK-9	Xylitol-9.5mg	9.5	1582268	10	11.68	1798844	1659580
10	BK-10	Xylitol-37.5mg	37.5	1533237	10	11.32	1728392	1087418
11	BK-11	Xylitol-150mg	150	1251579	10	9.24	1627455	492000
0	**Blank-4**	**Air Blank**		**0**	**0**	**0**	**0**	**0**
12	BK-12	S. Mutans E. Erytl-1.25mg	1.25	148546	10	1.10	0	137743
13	BK-13	S. Mutans E. Erytl-5mg	5	117115	10	0.86	0	167627
14	BK-14	S. Mutans E. Erytl-19mg	19	207706	10	1.53	60863	172709
15	BK-15	S. Mutans E. Erytl-75mg	75	178909	10	1.32	92606	190880
0	**Blank-5**	**Air Blank**		**0**	**0**	**0**	**0**	**0**
16	BK-16	S. Mutans E. Xyl-2.5mg	2.5	165235	10	1.22	41527	114900
17	BK-17	S. Mutans E. Xyl-9.5mg	9.5	181382	10	1.34	67282	149012
18	BK-18	S. Mutans E. Xyl-37.5mg	37.5	144051	10	1.06	94859	184817
19	BK-19	S. Mutans E. Xyl-150mg	150	198161	10	1.46	24503	131880

Table 1: Microbiome metabolites modification with polyols results table. Samples 1 through 3 show the background intensities of acetic acid, ethanol, and acetoin in control media with 10% sucrose, *S. mutans* negative control, and *S. mutans* positive control, respectively. Samples 4 through 7 have various amounts of erythritol and samples 8 through 11 have various amounts of xylitol added to the growth media. Samples 12 through 15 have both erythritol and *S. mutans* added to the growth media. Samples 16 through 19 have xylitol and *S. mutans* added to the growth media.

Discussion

Modification of the microbiome metabolites with polyols, or possibly the diet in general, has greater effects than previously appreciated. Research into epigenetic effects, the response of the genome to environmental factors, including the influence of SCFAs has greatly increased. Therefore, significant study of the microbiome, the microbiome effect epigenetically, and the modification of the microbiome via polyols, deserves intense interest. In this pilot study, we analyzed the effects of polyols on only one pathogen, but the effect was demonstrative.

In humans, the gut microbiota plays an important role in many functions, such as modulation of the immune system, production of vitamins and amino acids, the detoxification of harmful chemicals, and the breakdown of dietary fiber into short chain fatty acids. In this study, we examined the role that *S. mutans* may play in the production of short chain fatty acids in vitro, and how the changing environment (media with polyol added) has an impact on what types of SCFAs are produced. When a strain of *S. mutans* was grown with sucrose, it produced different SCFAs than when grown with the polyol erythritol. Most notably, when grown with erythritol, this strain no longer produced propionic acid.

By shifting production away from propionic acid, the erythritol environment allows other SCFAs to dominate amongst the metabolites of *S. mutans*. Propionic infusions into adult rat cerebral ventricles produces behaviors associated with Autistic Spectrum Disorder (A.S.D.) [32] and produces reversible repetitive dystonic behaviors, hyperactivity, turning behavior, retropulsion, caudate spiking, and the progressive development of limbic kindled seizures, coupled with neuroinflammatory, metabolic and epigenetic changes suggesting that it has central effects [33,34]. MacFabe, et al. also administered propionic acid subcutaneously and intra peritoneal finding very similar results [35,36]. Exposure of human lymphoblastoid cell lines to propionic acid elicited an atypical immunologic response [37,38]. On the other hand, propionic acid also has positive health effects with adults, such as anti-obesity, anti-inflammatory, and cholesterol lowering effects [39]. Calcium propionate has been utilized as a food preservative although the use appears to be decreasing. A large fast food restaurant chain recently announced discontinuing calcium propionate due to concerns over behavioral changes in children consuming calcium propionate preserved bread [40].

Additional laboratory study is required to test other species besides *S. mutans*, specifically the propionic producing *Clostridium histolyticum* and *bolteae*. By adding polyols to the diet, we could potentially shift the SCFA production to decrease the amount of propionic acid produced. Various low refined carbohydrate diets may help with ASD by reducing the substrates needed for SCFA production, and the supplementation of foods high in complex fibers may exert a therapeutic response in children by preferentially increasing the production of another SCFA, butyrate, over the production of propionic acid [41].

Short-Chain Fatty Acids (SCFA) formed by microbial fermentation have an important effect on colonic health [42,43]. Butyrate particularly has an important role in the metabolism and normal development of colonic epithelial cells and has been demonstrated to be protective against cancer and ulcerative colitis [44]. Butyrate is considered to be a preferred energy source for colonic epithelial cells and plays an important role in maintaining colonic health in humans. In a study of the colonic bacteria by Barcenilla et al, fifty percent of the butyrate-producing isolates were net acetate consumers during growth, but only 1% of the 239 non-butyrate-producing isolates consumed acetate [45]. Acetate would then seem to be an important precursor to butyrate production, a health benefit. However, too much acetate from bacterial production may promote metabolic syndrome [46]. Butyrate is essential for colonic health and has been shown to inhibit growth and induce apoptosis of colonic tumor cell lines [47], and could therefore be used for cancer treatment. An altered gut microbiome has been shown to increase SCFA production of acetic acid which will activate the parasympathetic nervous system, increase glucose-stimulated insulin secretion, increase ghrelin secretion (the hunger hormone), and contribute to hyperphagia and obesity [48]. Therefore, acetic acid could be targeted by therapeutics to reduce obesity.

The data already accumulated in regards to the therapeutic use of polyols encourages additional research into their microbiome metabolite shifts. The shift away from production of propionate could be of extreme importance, as research has clearly implicated propionate as a potential potentiator of A.S.D. symptoms [49,50]. The use of polyols to treat dental diseases has proven the safety of both xylitol and erythritol, not even considering the other positive side-effects such as the lowering of blood pressure, triglycerides and LDL-Cholesterol [51]. The oral microbiome of patients diagnosed with autism spectrum disorder also demonstrate an altered oral microbiome, with an increase in pathogens, such as, Streptococcus, that are susceptible to polyol therapy [52]. Knowing all this should reduce hesitancy in initiating animal then controlled human studies on microbiome shifts, metabolites shifting, and behavioral expressions using polyols.

Conclusions

Constituents of media, such as supplemental polyols, effect the bacterial metabolite production of *Streptococcus mutans* in vitro. Additional laboratory study is in progress testing other species, specifically the propionic producing genus Clostridia, specifically *Clostridium bolteae* and *Clostridium histolyticum* for the SCFA metabolite production, and the shift in SCFA production with the addition of polyols.

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