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Inhibition of Autism Spectrum Disorder Associated Bacteria and *C. difficile* by Polyols

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Abstract

Objectives: To determine the effectiveness of erythritol and xylitol in the inhibition of gut bacteria possibly associated with Autism Spectrum Disorder (ASD) and *Clostridium difficile* Infection (CDI).

Methods: Seven bacterial strains associated with ASD, or with CDI and a control probiotic were tested for polyol inhibitory activity: *Clostridium histolyticum*, *Bacteroides vulgatus*, *Bifidobacterium longham*, and two strains each of *Clostridium bolteae* and *difficile*. Each strain was grown in brain heart infusion/sucrose media with polyol concentrations varying from 0% to 15% for erythritol and 0% -30 % for xylitol. Growth of *Clostridium histolyticum* and *Bifidobacterium longham* was measured after 24 hours while all other strains were evaluated at 48 hours to permit additional growth. Optical density was measured using a spectrophotometer and the plates were read at 620 nm.

Results: All strains had results indicating polyol inhibition of growth. *Clostridium histolyticum (Chis)*, *Bifidobacterium longham (Blof)*, and both *Clostridium bolteae* (Cbol) strains showed reduced growth with increasing polyol concentration with an inflection point of about 4% for both xylitol and erythritol (complete or near complete inhibition relative to control wells). *Bacteroides vulgatus* (Bvul) grew very lightly in the BHI/sucrose. This strain has visible growth but very low OD values. Inhibition of growth with increasing polyol concentrations was observed but assessing the polyol inhibition break point was difficult with this strain.

Conclusions: Xylitol and erythritol at sufficient concentrations were able to inhibit the growth of bacterial strains that have been associated with the development of Autism Spectrum Disorder in recently published studies.

Keywords: Autism Spectrum Disorder, polyol, Bacterial strains, Optical density.

Abbreviations: ASD-Autism Spectrum Disorder, CDI-*Clostridium Difficile* Infection, Cbol- *Clostridium bolteae*.

Introduction

Polyols have been used for decades as a substitute for sucrose [1-4]. The most commonly used polyols for consumption are sorbitol, mannitol, xylitol, erythritol, maltitol, lactitol and isomalt [5]. Besides having fewer calories than regular sucrose, i.e., table sugar, polyols have other reported health benefits, especially in regards to oral health [6]. Well publicized studies showing the effectiveness of xylitol at reducing dental disease have been reported for decades, all with results demonstrating safety and effectiveness [7-9]. The well-publicized "Turku" and the "Belize" studies reported on the caries reduction by xylitol, with xylitol being more effective than sorbitol [10,11]. Xylitol chewing gums, toothpastes, lollipops, candies and mouth rinses are all part of a complete dental oral hygiene program [12].

Erythritol and xylitol are polyols that repeatedly have been demonstrated to possess anti-cariogenic and anti-periodontal disease properties [13]. Polyols (particularly the non-hexitol alditols or "sugar alcohols" erythritol and xylitol) have been found effective in inhibiting the transition to and maturation of biofilms from planktonic cells [14]. Xylitol clearly inhibits the formation of mixed species biofilms, in vitro [15].

Erythritol suppresses the maturation of biofilms and contributed to a healthier oral ecosystem [16]. Polyols can suppress the growth and virulence expression of mixed bacterial biofilms. Erythritol was the most effective polyol in suppressing the growth and organization of dental pathogens. Erythritol also exerted inhibitory effects on several pathways reduced growths through DNA and RNA depletion, attenuated extracellular matrix production and alterations of dipeptide acquisition and amino acid metabolism [17].

The bacteria associated with Autism Spectrum Disorder have been reported in the literature, with similar results independent of research institution and locality [18]. Autism Spectrum Disorder (ASD) has been linked to propionic acid producing bacterial species, such as, *Clostridia bolteae* and *Clostridia histolyticum* [19-22].

Conversely the presence of Clostridia sporogenes could help protect against ASD by combining propionic acid with indole to produce 3-Indole Propionate, a neural protective metabolite, thereby neutralizing the epigenetic effect of propionic acid [23-25]. It has been theorized that the absence of *C. sporogenes* in the soil is related to the use of glyphosate, known by the trade name Roundup [18]. Absence of *C. sporogenes* in the soil and the environment could possibly shift the maternal microbiome, resulting in epigenetic changes in the fetus or

infant. Bacteroides vulgatus also has been implicated in ASD as reported in the Frontiers in Microbiology by Coretti et al. [26].

Clostridia difficile (Cdif) is a gram-positive bacterium that is implicated in antibiotic-associated diarrhea. The relatively recent emergence of a newer hyper-virulent North American strain (NAP1) has been associated with the increase in incidence and severity of *C. difficile* infections (CDI) over the last decade [27]. Antibiotic overuse remains the leading risk factor for *C. difficile* infection. Several classes of antibiotics such as penicillins, cephalosporins, fluoroquinolones, and clindamycin have been implicated in causing CDI.

Besides antibiotic usage, other risk factors are reported to include advanced age, chemotherapy, use of proton pump inhibitors, chronic renal disease, chronic liver disease and malnutrition [28,29]. Treatment options include discontinuing the causative antibiotic and administering either vancomycin or fidaxomicin. Another option is fecal transplantation, the process in which feces from a healthy donor are transplanted into the intestinal tract of a person with the disrupted microbial balance. This protocol has reported an 80% to 90% success rate in reducing the recurrence of *C. difficile* infections [30]. There remains some opposition to Fecal Transplantation Therapy due to the basic nature of the procedure and potential complications [31]. A simpler, safer and "cleaner" technique would be more appealing to patients and clinicians.

Materials and Methods

Bacterial isolates and media: *C. bolteae* and *C. histolytica* strains were kindly provided by Dr. Emma Allen- Verco PhD. (University of Guelph/Canada). *B. vulgatis* (8482) and *B. longum* (15707) were obtained from the American Type Culture Collection (ATCCC/Manassas Va.). *C. difficile* strains 5555 and 5557 were provided by Dr. Larry Kociolek MD (Lurie Children's Hospital, Chicago, IL). All studies used a basal media of Brain Heart Infusion broth supplemented with 2% sucrose (BHI/Suc). Polyols were prepared separately at high concentrations in BHI/Suc for assay plate preparations. Xylitol was added to 60% (w/v) and Erythritol was prepared at 30% (w/v) in BHI/Suc. These polyol levels were the maximum achievable based on solubility. Final media preparations were sterilized and placed in an anaerobic chamber for at least 2 hours after preparation to cool and remain in a reduced state.

Assay Procedures

Assays were prepared in the anaerobic chamber. 96 well plates were employed with each test preparation in triplicate wells by adding 100 mcL of BHI/Suc at 2x concentration to all test wells. Bacterial preparations were made in BHI/Suc adjusted to a Macfarland standard concentration of 0.5. Final assay inocula of each strain with a further 1:100 fold dilution. 100 mcL of bacterial inocula was added to each test well with or without a polyol. Plates were incubated anaerobically for 24 or 48 hours and terminated when bacterial growth reached a easily visible level in control wells. Plate were then transferred to a plate spectrophotometer and read at 620 nm wavelength. Mean OD values for each well were calculated and OD values vs. polyol concentration were plotted.

Results

Seven strains were tested for polyol inhibitory activity *C. histolyticum*, *B. vulgatus*, *C. bolteae* (x2), *C. difficile* (x2), and *Bifidobacterium longham*. All strains grew to variable bacterial density levels. B. vulgatus had the poorest growth but still had measurable mean OD values to suggest polyol activity. Detailed OD values vs. polyol concentration are plotted as follows with relative inhibition inflection points **(Figures 1-7)**.

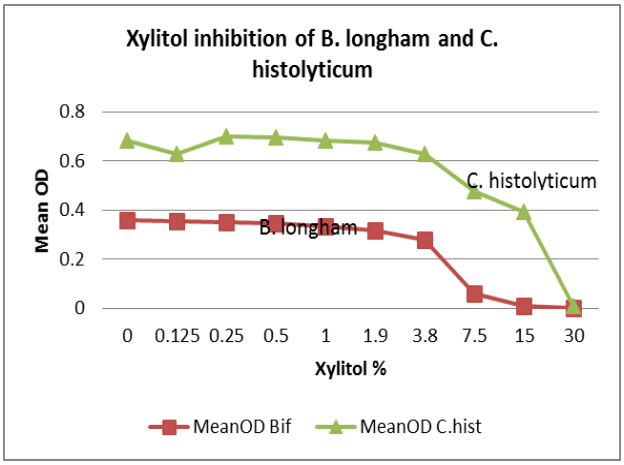

Figure 1: Xylitol begins inhibiting *C. histolyticum* with only a 2% concentration. The probiotic is also inhibited and supplementation may be advised.

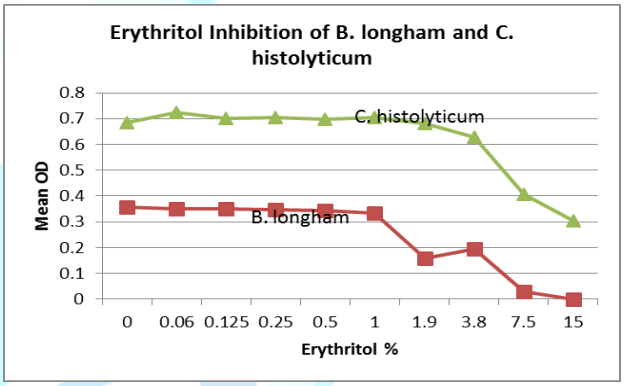

Figure 2: Erythritol inhibits at around a 2% concentration but inhibits the probiotic more that xylitol.

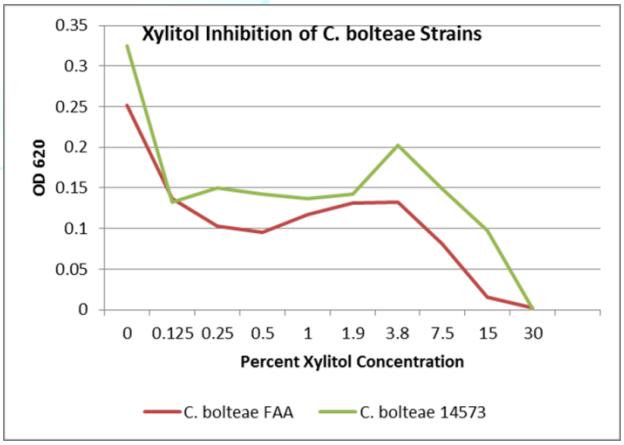

Figure 3: *C. bolteae* strains inhibited by a very low concentration of xylitol, but less so at a 2% level.

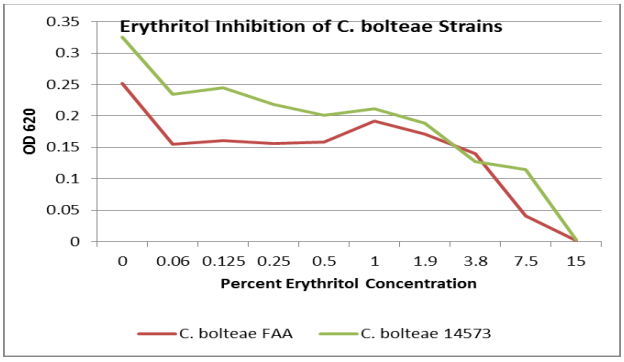

Figure 4: Erythritol inhibits the *C. bolteae* strains at a very low concentration and in a more linear path than xylitol.

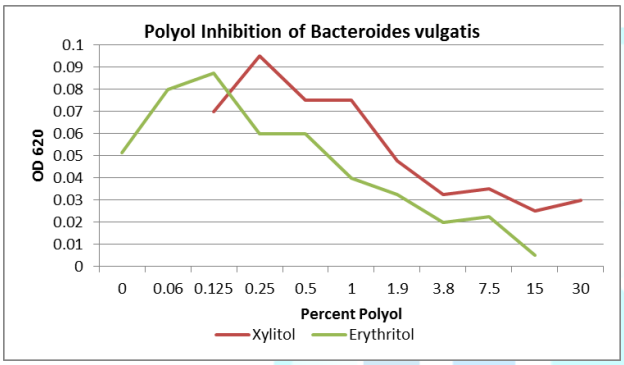

Figure 5: Both erythritol and xylitol inhibits *B. vulgatis* at only a 0.25% concentration.

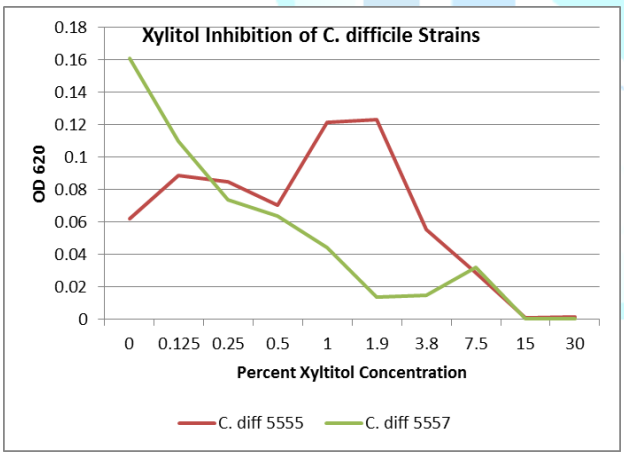

Figure 6: At 2% concentration, xylitol generally inhibit *C. difficile* strains, although *C.diff* 5557 is inhibit in a more linear manner and at a lower concentration.

Discussion

Erythritol inhibits ASD bacteria at a lower concentration than xylitol. Both polyols were capable of significant inhibition of the ASD associated bacteria, in addition to the inhibition of antibiotic resistant *C. diff* strains. Erythritol may inhibit *Bacteroides vulgatus* better than xylitol but additional studies with a more optimal media for *B. vulgatis* need to be performed.

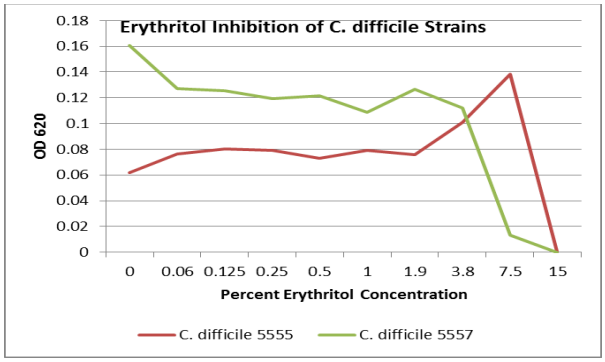

Figure 7: Erythritol seems to inhibit *C. diff* only after reaching a higher concentration. Most dental products contain 20% xylitol for effectiveness. There are no popular dental products with a high erythritol concentration, although some commercial drinks use erythritol as a sweetener.

However, Xylitol should be considered as a treatment for *C. difficile* infection due to its low cost and availability. In addition, xylitol and erythritol are considered safe food additives with decades of use in the prevention of oral diseases, such as periodontal disease and dental cavities.

Autism spectrum disorders are likely caused by a combination of microbiome, environment, and the epigenetic interaction [32-34]. Recent research shows that more than 50% of children with autism have GI symptoms, food allergies, and maldigestion or malabsorption issues [35].

Propionic acid is used as a food additive and is also a bacterial byproduct. Propionic acid uptake may be related to lack of the bacterial gluten metabolizers and resultant leaky gut. Elimination of calcium propionate as a bread additive/preservative may be beneficial in reducing the behaviors associated with ASD [36]. Shifting the oral and gut microbiome with polyols may also be successful in reducing behaviors associated with ASD. More research, large well-designed clinical trials are indicated for protocols illuminating therapies effective with reducing the symptoms of ASD [37].

Conclusion

Xylitol and erythritol at sufficient concentrations were able to inhibit the growth of bacterial strains that have been associated with the development of ASD. Further research into the use of polyols for the treatment and possible prevention of ASD is recommended. Large clinical trials with patients that are correctly diagnosed with ASD then treated with xylitol supplementation and the resultant effects on behavior should be carefully explored. In addition, the uses of polyols to treat *C. difficile* infections also require clinical trials.

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