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Impact of Carbohydrates on the Aggregation of Probiotic Bacteria

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ABSTRACT

Probiotic bacteria can be beneficial to the gastrointestinal tract in the human body in numerous ways. Autoaggregation may play a key role in allowing probiotics to prevent pathogenic organisms from colonizing the intestinal system. Currently, scientific research does not account for the extent in which the autoaggregation capacities of probiotics may be influenced by carbohydrates. In this experiment, nine carbohydrate sources, including those with prebiotic qualities, were applied to eighteen strains of bacteria of the *Lactobacillus* genus. The experiment evaluated the autoaggregation abilities of the lactobacilli strains exposed to the carbohydrate treatments. Generally, no carbohydrates stimulated the autoaggregation of most strains of lactobacilli. However, experimental results confirmed the rapid autoaggregation of *Lactobacillus acidophilus* La-5 exposed to treatments of 2'-fucosyllactose. These experimental results are relevant in understanding how carbohydrates may indirectly impact how probiotics can prevent pathogens from colonizing the gastrointestinal tract within the human body.

INTRODUCTION

Scientific research concerning the extent to which carbohydrates impact the aggregation of probiotic bacteria is lacking. Probiotics are traditionally regarded as "live microorganisms that when administered in adequate amounts confer health benefits on the host" (Bertazzoni et al., 2013). Scientific studies have confirmed the extent to which probiotic bacteria metabolize carbohydrates, but scientific evidence indicating how carbohydrates physically affect the aggregation of probiotic bacteria is virtually non-existent. Probiotic bacteria, including strains of *Lactobacillus*, can stimulate and regulate growing populations of bacteria beneficial to the vitality of the gastrointestinal tract of the human body. Research indicates that certain strains of *Lactobacillus* are known to convey probiotic benefits upon human consumption; they are commercially added to dairy products and dietary supplements. Autoaggregation may enable probiotic bacteria to prevent pathogenic organisms from colonizing along epithelial cells within gastrointestinal systems (Goh & Klaenhammer, 2010).

Throughout the intestinal system, beneficial intestinal bacteria, including strains of lactobacilli, ferment oligosaccharides, short to medium length carbohydrate polymers. Prebiotics are conventionally defined as "non-digestible but fermentable oligosaccharides that are specifically designed to change the composition and activity of the intestinal microbiota with the prospect to promote the health of the host" (Blaut, 2002). Fructose and galactose are the main monosaccharides that compose fructooligosaccharides (FOS) and galactooligosaccharides (GOS), respectively, typically within the dietary fibers of food products. The literature documents much evidence for the benefits of prebiotics, including stimulated growth of lactobacilli within the human intestinal tract (Fanaro et al., 2005).

Potentially instrumental in promoting the growth of intestinal bacteria, prebiotics are nutritional ingredients added to some brands of infant formula. Infant formula

contrasts from human milk in that its composition is based upon cow's milk, lacking oligosaccharides vital to infantile intestinal health. Human milk oligosaccharides (HMOs) potentially have the ability to inhibit the adhesion of pathogenic bacteria to epithelial cell surfaces along the intestinal tract (Newburg et al., 2005; Barile & Rastall, 2013). Since formula-fed infants are not exposed to the HMOs that breast-fed infants are, they are disadvantaged in producing strains of beneficial intestinal bacteria, such as lactobacilli, and potentially exposed to intestinal pathogens.

Ultimately, prebiotics may potentially affect the means in which bacterial aggregation can prevent the colonization of pathogens along intestinal epithelial surfaces. Research has not yet accounted for the mechanisms of interactions that occur between lactobacilli and carbohydrates. We hypothesized that different carbohydrate sources affect the autoaggregation of different strains of lactobacilli. The following experiment examined the range of autoaggregation abilities of different strains of lactobacilli that are exposed to different carbohydrate sources. Evaluating the range of autoaggregation abilities of lactobacilli strains, as a whole, is crucial in understanding the autoaggregation capacities of selected probiotic strains. Future implications of this experiment may stimulate in-depth research concerning the dietary effects of known probiotics and further the engineering of prebiotic supplements as nutritional components of infant formula.

LITERATURE REVIEW

Probiotic organisms, upon consumption, positively affect the overall health of the host in numerous ways. The following are among the multiple genera of yeasts and bacteria that can act as probiotic organisms: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium*, *Saccharomyces*, and *Enterococcus*. Probiotic microorganisms belonging to the *Lactobacillus* genus consist of several strains of bacteria that produce lactic acid via anaerobic fermentation. Probiotic strains of lactobacilli can promote sustained populations of commensal bacteria that inhabit the gastrointestinal system (Servin & Conconnier, 2003). Products containing certain strains of lactobacilli as commercial probiotics are commonly administered as dietary supplements; the strains *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* NCFM, *Lactobacillus casei* Shirota, and *Lactobacillus reuteri* MM53 are examples of lactobacilli that exhibit probiotic qualities and are commonly used as dietary supplements (Reid, 1999). Strains of lactobacilli effectively function as probiotics based upon their ability to thrive in the intestinal system, produce lactic acid, adhere along epithelial cell surfaces, and prevent the colonization of pathogenic organisms along the gastrointestinal tract**.**

Universally notorious for causing intestinal infection and diarrhea, *Campylobacter jejuni* is a bacterial pathogen that initially adheres to the mucus-lined surfaces along the intestinal tract. A compilation of studies of Mexican children revealed that breast-feeding proves to be a viable solution for preventing campylobacter diarrhea (Ruiz-Palacios et al., 2003). Human milk contains large amounts of complex oligosaccharides, which can inhibit the colonization of *Campylobacter jejuni* in the intestinal system. HMOs are polymers consisting of two to twenty monosaccharides, or simple sugars; HMOs can function as receptors for the H(O) blood antigen. HMOs prevent colonizing *Campylobacter jejuni* by regulating the amount of H-2 milk-specific antibodies available for pathogens to bind with. Observing that campylobacter binding to H-2 antigens causes infection, an experiment hypothesized that campylobacter's host receptor may contain carbohydrate components that enable them to receive blood group antigens (Ruiz-Palacios et al., 2003).

According to the hypotheses set by Ruiz-Palacios et al., three sets of experiments were conducted. First, an experiment was conducted to determine whether fucosylated oligosaccharide fractions of human milk inhibit campylobacter from adhering to epithelial cells *in vitro.* Second, an experiment confirmed the nature of the host receptors. The third set of experiments determined whether fucosylated HMOs inhibit *C. jejuni* in the human intestinal system *in vivo* and whether the transfer of an H(O) antigen gene in mouse pups prevents colonization.

As the initial sets of experiments consisted of a mouse model, the experimental results indicated that mice orally administered dosages of human milk oligosaccharides had significantly low intestinal colonization of *Campylobacter*. The second set of experimental results revealed that fractions of neutral

HMOs and 2'-fucosyllactose (2FL) inhibited higher percentages of pathogenic campylobacter strains than non-pathogenic campylobacter strains. The final experimental results indicated that mice pups exposed to HMOs, which functioned as antigen-specific receptors, were less impacted by inoculated campylobacter than pups that were not exposed to HMOs. The experiment concluded that fucosylated structures are potentially critical features of human milk. It is presumed that 2FL competes with H-2 antigens by binding to *Campylobacter*. The experiment warrants further research on how the H-2 antigen functions as prerequisites for colonization of campylobacter. Prebiotic carbohydrates are manufactured to imitate the abilities of HMOs (Espinosa et al,. 2007), such as potentially preventing pathogens from colonizing the gastrointestinal tract.

Our study aims to acquire knowledge about the aggregation of lactobacilli strains rather than strains of campylobacter. Select strains of lactobacilli can form aggregates and demonstrate probiotic characteristics. Examining how such probiotic strains autoaggregate may provide more in-depth knowledge about how probiotics aggregate overall. Scientific evidence indicates potential prevention of intestinal infections when probiotic bacteria bind with pathogenic organisms; scientific evidence has attempted to link the ability of bacterial autoaggregation with the tendency of bacteria to coaggregate. Based upon this association with coaggregation, lactobacilli bacterial autoaggregation may also play a role in inhibiting pathogens from colonizing the intestinal tract; thus, it would be critical to examine what factors may influence autoaggregation. Carbohydrates, for instance, were factors of our experiment that were tested to determine their potential effects on lactobacilli autoaggregation *in vitro.* Autoaggregation of probiotic bacteria may reveal knowledge concerning how probiotics function when exposed to other factors, aside from pathogenic organisms.

Prebiotics are non-digestible oligosaccharides that exit the small intestines to be fermented by intestinal bacteria, including certain strains of lactobacilli, found within the colon (Gibson et al., 2004)**.** The fermentation of prebiotic carbohydrates contributes to the growth of lactobacilli strains, altering the intestinal microbiota. Glucose, galactose, maltose, fructose and lactose are examples of saccharides that compose oligosaccharides. Commonly manufactured prebiotics include FOS, GOS, polydextrose (PDX), and inulin. Dietary fibers within food products typically contain prebiotic carbohydrates such as FOS and GOS. Scientific research was conducted to confirm evidence of the benefits conveyed by prebiotics, including the stimulated growth of lactobacilli within the human intestinal tract (Fanaro et al., 2005).

FOS and GOS have been shown to promote growth in select strains of lactobacilli in infant intestinal tracts. Prebiotics, such as FOS and GOS, are added to some infant formulas as substitutes for HMOs innate to breast milk (Knol et al., 2005). HMOs are instrumental in promoting the growth of intestinal lactobacilli via fermentation (Ruiz-Palacios et al*.,* 2003). HMOs also impact the inhibition of intestinal pathogens. In several *in vitro* experiments, formulated mixtures of 90 % short-chain galactooligosaccharides and 10 % long-chain fructooligosaccharides were intended to emulate the properties of neutral HMOs (Fanaro et al., 2005).

Haarman and Knol performed two experiments where mixtures of long-chain FOS and short- chain GOS were supplemented in infant formula administered to term and preterm infants (Haarman & Knol, 2005). Collectively, the results from each experiment indicated large populations of lactobacilli and bifidobacteria had accumulated in the gastrointestinal tract of the term and preterm infants. Each experiment concluded that when supplemented in infant formula, long-chain FOS and short-chain GOS had the ability to emulate properties of HMOs innate in breast-milk. A major property of HMOs involves stimulating the growth of massive populations of bifidobacteria and strains of lactobacilli that potentially confer benefits upon infant immune and intestinal system. Aside from FOS and GOS, inulin is another carbohydrate whose prebiotic capabilities have been tested in numerous experiments. For instance, the ability of inulin to generate the growth of beneficial, intestinal bacteria has been studied in several experiments. In a 2004 study completed by Moro et al., experimental results witnessed increasing populations of bifidobacteria and lactobacilli species with the intestinal system of infants exposed to mixtures of inulin and GOS (Moro et. al, 2004). The experiment concluded that the mixture of GOS and inulin, alternatively manipulated instead of FOS, had the ability to stimulate lactobacilli strains that potentially conveyed nutritional benefits upon infant intestinal systems.

Additionally, the experimental results each involved the reduction of pathogenic organisms that coincided

with the increase of beneficial intestinal bacteria in the infantile intestinal system (Haarman & Knol, 2005). Each of these experiments reported reduced populations of pathogenic Clostridia associated with increasing bifidobacteria along the gastrointestinal tract. These experiments concluded that FOS and GOS, supplemented in infant formula, may prove critical in preventing the colonization of intestinal pathogens similarly to HMOs innate to breast milk. Thus, it can be reasonably deduced that prebiotics can help g row beneficial organisms and prevent colonizing pathogens in the gastrointestinal system. However, my experiment sought gain scientific evidence indicating how carbohydrates, regardless of prebiotics or non-prebiotics, affect aggregation of probiotic bacteria. Bacterial aggregation, among other mechanisms of probiotics, was of interest in our experiment. Specifically, the potential effect of carbohydrates on the autoaggregation probiotic lactobacilli was most relevant to our study.

In order to colonize and infect the intestinal systems, pathogenic organisms initially adhere along epithelial cell surfaces along the gastrointestinal tract. Adhesion to epithelial cell surfaces enables probiotic bacteria to thrive in the intestinal system, allowing them to potentially inhibit pathogens from colonizing the gastrointestinal tract. For this reason, adhesion is often regarded as an essential requirement in selecting effective probiotics (Holzapfel & Schillinger, 2002). Additionally, bacterial aggregation is a favorable trait in selecting effective probiotics; some probiotic bacteria can aggregate, or form bacterial clusters, which potentially excludes pathogens from adhering to mucous surfaces in the intestinal tract. Coaggregation, or the clustering of two or more different types of bacteria, can involve probiotic bacteria binding to pathogenic bacteria. Autoaggregation, or uniform clustering, of only probiotic bacteria may assist in preventing pathogenic adherence to epithelial surfaces and colonization of the gastrointestinal tract.

Numerous bacterial strains of the *Lactobacillus* genus can autoaggregate. While scientific research vaguely accounts for the mechanisms of lactobacilli aggregation, scientific evidence attributes aggregation to the interactions between components of the cell surface and secreted proteins. The adhesion abilities of lactobacilli strains are relative to the hydrophobic components on their cell surfaces; yet, scientific data has revealed discrepancies indicating these hydrophobic components may impact the autoaggregation of lactobacilli strains (Goh & Klaenhammer, 2010). Collectively, adhesion, hydrophobicity, autoaggregation and coaggregation are all factors that may enable probiotic bacteria to inhibit pathogenic intestinal colonization.

An experiment was conducted by to determine whether bacterial aggregation and cell surface hydrophobicity of dairy *L. plantarum* strains were accurate indications of their adhesion abilities and potential for competitive exclusion of intestinal pathogens (García-Cayuela et al., 2014). Among one-hundred twenty-six *L. plantarum* isolated from raw goat milk, five of the fourteen rapidly autoaggregated strains expressed phenotypes for dense aggregation. In the coaggregation assays, all *L. plantarum* strains and pathogens experienced coaggregation, yet the extent of coaggregation was dependent of the lactobacilli and pathogenic strains manipulated. It was concluded that strains with the highest autoaggregation abilities and expressed aggregation phenotypes were the most likely to coaggregate. Initiated by negative charges, hydrophobic components on cell surfaces directly affect the cellular adherence abilities, varying among bacterial strains.

However, it concluded that cellular hydrophobicity was not the most accurate means of indicating the autoaggregation of lactobacilli strains. The experiment concluded that lactobacilli strains' adhesion to epithelial cells were not always correlated with the aggregation phenotype, coaggregation abilities, and hydrophobic properties of the cell's surface (García-Cayuela et al., 2014). Aggregation abilities and hydrophobicity may not be as significant as other factors involved in adhesion and pathogenic competition. In regards to adhesion, they are potentially more relevant to the survival of lactobacilli, especially probiotic strains, within the gastrointestinal tract.

METHODOLOGY

Bacterial strains and growth conditions

Eighteen strains of *Lactobacillus* were manipulated in this experiment (Table 1). All tested bacteria were stored at -80 ºC prior to use. Lactobacilli were grown in de Man, Rogosa, Sharpe (MRS) broth under anaerobic atmosphere consisting of 90 % N_2, 5% CO_2, and 5 % H_2, at 37 ºC for 24 hours.

Table 1
Strains of *Lactobacillus*.

MJM*	Organism	Strain
4	*Lactobacillus gasseri*	ATCC 3333
7	*Lactobacillus acidophilus*	NCFM
9	*Lactobacillus rhamnosus*	ATCC 53103
13	*Lactobacillus johnsonii*	ATCC 11506
39	*Lactobacillus acidophilus*	ATCC 4356
53	*Lactobacillus rhamnosus*	ATCC 9595
73	*Lactobacillus salivarius* subsp. *salivarius*	ATCC 11741
89	*Lactobacillus johnsonii*	La-1
90	*Lactobacillus plantarum*	LP-66
96	*Lactobacillus acidophilus*	La-5
108	*Lactobacillus fermentum*	CECT5716
110	*Lactobacillus reuteri*	MM53
149	*Lactobacillus casei*	LB6
155	*Lactobacillus plantarum*	LB12
206	*Lactobacillus crispatus*	CC1-1
207	*Lactobacillus crispatus*	JCM5810
208	*Lactobacillus gallinarum*	ATCC 33199
209	*Lactobacillus helviticus*	CNRZ32

*MJM refers to the strain number from the culture collection of Prof. Michael J. Miller.

Treatments with Carbohydrate Competitors

Ten treatments were applied to each of eighteen strains of lactobacilli. The following nine carbohydrates were utilized at concentrations of 10 mg/mL: inulin, FOS, GOS, lactose, maltodextrin, PDX, glucose, 2FL, and mannose. Water was used as a control treatment. Aliquots of each strain (0.5 mL) were prepared in 1.5 mL microcentrifuge tubes. Cells were harvested by centrifugation at 3000 x g for 5 minutes, followed by the removal of the MRS supernatant and re-suspension in 0.5 mL acetate buffer (50 mM, pH 4.0) via brief vortexing. Equal volumes of each competitor were added to their respectively labeled test tubes containing the eighteen strains. Each of the sets of test tubes was briefly vortexed, prompting additional suspension of the lactobacilli strains in contact with the carbohydrate sources. Strains were observed over 60 minutes, after which autoaggregation was assessed.

Qualitative Assessment

The visible extent of autoaggregation for the lactobacilli strains, or lack thereof, was qualitatively measured. In conjunction with autoaggregation, the pelleted formations of the bacterial cells were measured using an alphabetized scale. Positive (+) and negative (-) symbols were used to indicate the extent of autoaggregation visibly demonstrated by strains of lactobacilli. The letters, “A” through “D” were used to alphabetically categorize pellet formation, ranging from minimal formation of bacterial debris to compact portions of aggregated bacteria or loose pellet layers that exhibited uniform autoaggregation.

FINDINGS

Observations for the autoaggregation of each *Lactobacillus* strain are provided in Table 2. Some variability occurred in the control treatments from day to day, although carbohydrate treatments were consistent with the control with minor variation. This is best exemplified in the alternating patterns of autoaggregation MJM 39 on Day 1 and Day 3 and the autoaggregation of MJM 108 only on Day 2. MJM 9 autoaggregated in PDX faster than when exposed to the control treatment; however, replicated treatments did not result in MJM 9 autoaggregating faster in the PDX than in the control.

MJM 96 autoaggregated in the presence of 2FL faster than with the given control of the experiment; MJM 96 autoaggregated to a substantial extent more so when exposed to 2FL than when exposed to the experimental control on that given day. Non-aggregative strains showed no signs of autoaggregation in the midst of the carbohydrate treatments.

Table 2
Autoaggregation of lactobacilli in the presence of soluble carbohydrates.

	Day 1				Day 2					Day 3		
MJM*	CTRL	INU	FOS	GOS	CTRL	MAL	GLU	PDX	LAC	CTRL	2FL	MAN
4	+++ B	+++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B
7	+++ B	+++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B
9	D 0.5	D 0.5	D 0.6	D 0.7	D 0.3	D 0.5	D 0.3	+++ C	D 0.4	D 0.5	D 0.5	D 0.3
13	-	-	- A	-	-	-	-	-	-	-	-	-
39	++ B	++ B	++ B	++ B	-	-	-	-	-	++ B	++ B	++ B
53	- A	- A	- A	-	-	- A	+ A	- B	-	- A	- A	- A
73	-	-	-	-	-	-	-	-	-	-	-	-
89	+ B	+ B	+ B	+ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B	++ B
90	-	-	-	-	-	-	-	-	-	-	-	-
96	++ B	+ B	+ B	+ B	++ B	+++ B	+++ B	++ B	+ B	+ B	+++ B	+ B
108	-	-	-	-	++ B	++ B	++ B	++ B	+ B	-	-	-
110	-	-	-	-	-	-	-	-	-	-	- A	-
149	+++ C	+++ C	+++ C	+++ C	+++ B	+++ B	+++ B	+++ B	+++ B	D 0.2	D 0.3	D 0.2
155	+++ B	+++ B	+++ B	+++ B	+++ B	++ B	+++ B	+++ B	++ B	+++ B	+++ B	+++ B
206	-	-	-	-	-	-	-	-	-	-	-	-
207	-	-	-	-	++ B	++ B	++ B	++ B	+ B	-	-	-
208	+	+	+	+	++ B	++ B	++ B	++ B	++ B	++ B	+ B	+
209	+	-	+	-	-	-	- B	+	-	-	-	-

*MJM: refers to the strain number from the culture collection of Prof. Michael J. Miller
CTRL: control; INU: inulin; FOS: fructooligosaccharide; GOS: galactooligosaccharide; MAL: maltodextrin; GLU: glucose; PDX: polydextrose; LAC: lactose; 2FL: 2'-fucosyllactose; MAN: mannose
- : no visible aggregates were in the cell suspension
+ : small uniform aggregates were in suspension; sand-like grains of cells in small clusters seen with careful observation
++ : easily visible aggregates; formed clusters were distinct from supernatant or remaining suspension
+++: large obvious aggregates visible, may or may not leave some turbidity in the supernatant fluid
A: presence of a small pellet or powdery collection of cells that settled out; potentially debris or dead cells fallen out of suspension
B: a relatively tightly-packed pellet that generally forms rapidly upon aggregation of cells
C: a loose layer formed on the bottom of tube, indicative of aggregation in a loose network; some flocs remained in suspension
D: full aggregation indicated by no turbidity in supernatant above pellet; indicative of uniform aggregation of cells that slowly migrated down and formed even separation from the surface. Followed by a number indicating the volume (mL) of the cell pellet as it settled

No instruments were utilized to quantify the results, so it is understandable that the experimental observations demonstrated some variability between treatments due to the qualitative nature of the assessments used. Generally, the growth conditions and handling of lactobacilli may vary as experimental results were taken on different days; the variation of growth factors and handling of lactobacilli may have potentially influenced their capacities for autoaggregation. Mishandling of lactobacilli may have involved the mislabeling of different lactobacilli strains, pipetted disruptions of pelleted cells, and vortexing technique that created time lags in the autoaggregation of certain lactobacilli strains. In the case of MJM 39 and MJM 108, their autoaggregation most likely resulted from mislabeling of those bacterial samples or contamination in contact with the strains.

The autoaggregation of MJM 9, when exposed to PDX, occurred more rapidly than the strain's autoaggregation subject to the control treatment. The occurrence of MJM 9 to autoaggregate faster in contact with PDX rather than in the control treatment may have been determined based upon an erroneous observation of a mislabeled strain. Additional trials were most likely unable to replicate this rapid autoaggregation due to the

presence of potential contaminants. Regardless of MJM 9's phenotypic traits for aggregation, MJM 9 was not prompted to aggregate faster with PDX treatments than with control treatments in the replicated trials.

The autoaggregation of MJM 96 subjected to the control treatment occurred at a slower rate than the strain's autoaggregation in the presence of 2FL. Additional trials resulted in the autoaggregation of MJM 96 in contact with 2 FL at faster rates than the strain's autoaggregation in the presence of control treatments. These trials further confirmed the presence of more dense aggregates of MJM 96 in the concentrated 2FL treatments than those aggregates formed in the control treatments. As these replicated trials support the conclusion that 2FL promotes the aggregation of *L. acidophilus* La-5, further research is required to understand how 2FL interacts with this strain and what are the implications that their interactions have on the capacities to autoaggregate in in vivo systems.

The experiment manipulated the following three strains of *Lactobacillus acidophilus*: NCFM (MJM 7), ATCC 4356 (MJM 39), and La-5 (MJM 96). While each strain was exposed to the 2FL treatments, MJM 96 autoaggregated to a more compact extent than MJM 7 or MJM 39. Each of the strains formed tightly packed pallets that resulted from immediate autoaggregation. However, compared to MJM 7 and 39, MJM 96 formed large, clearly defined aggregates within the turbid supernatant fluid; the rapid autoaggregation of MJM 96 subject to 2FL treatments was confirmed in three trials. These experimental results indicated that carbohydrates can have more than just species-specific impacts on autoaggregation. As demonstrated by the autoaggregation of the probiotic MJM 7, 39, and 96 strains in 2FL treatments, carbohydrates may be able to assert strain-specific affects the on bacterial autoaggregation. This conclusion is supported by the resulting immediate autoaggregation of MJM 96 when subject to 2FL treatments.

As the extent of autoaggregation is dependent on a strain's aggregation capacities, non-aggregative strains did not present any indication of being prompted to autoaggregate by any of the carbohydrate treatments. Non-autoaggregative qualities may be expressed by lactobacilli considered to have probiotic effects. Regardless of whether lactobacilli were non-probiotic or derived from commercial probiotics, the prebiotic carbohydrates did not promote autoaggregative responses from known aggregative and known non-aggregative strains of *Lactobacillus*. This suggests that carbohydrates are unable to induce the autoaggregation of lactobacilli, regardless of probiotic or non-probiotic qualities are present.

However, there are several limitations in the experimental design that may limit the applicability of these conclusions. A major limitation of the experiment involved the use of only 18 strains of lactobacilli to determine how carbohydrates collectively affect multiple strains of lactobacilli, in terms of autoaggregation. The autoaggregation, or lack thereof, of these select 18 strains of lactobacilli were used for generalizing how carbohydrates potentially influenced the autoaggregation of all probiotic lactobacilli strains. The range of autoaggregation responses, prompted by the prebiotic carbohydrates, were used for evaluating the extent in which all prebiotics are capable of influencing bacterial autoaggregation. Furthermore, a significant limiting factor involved the environment where the experiment occurred. As the experiment tested the autoaggregation for the lactobacilli strains exposed to carbohydrate treatments, the resulting aggregates formed clusters while exposed to in vitro conditions. In the laboratory setting, the lactobacilli strains and carbohydrates were contained in isolated environments until manipulated for experimental purposes. The autoaggregation of some strains may have been more or less likely to occur due to the presence or absence favorable conditions of the in vitro setting. Environmental conditions that are potentially conducive for the autoaggregation or even bacterial growth of certain lactobacilli strains include optimal pH measurements and temperatures, which were not manipulated in this study. The extent in which the bacteria autoaggregated in these conditions were projected for how these strains would autoaggregate in the gastrointestinal system.

Experimental results from *in vitro* testing would not be indicative of any given strain's ability to autoaggregate along epithelial surfaces along the intestinal tracts. In *in vivo* settings, strains of beneficial intestinal bacteria, or those originating from the *Lactobacillus* genus, would have more direct contact to numerous microorganisms that also inhabit the microbiota of the human gut. As such microorganisms were not present during the experimental trials, there

are no documented results that can account for how these such organisms may impact bacterial autoaggregation. These intestinal organisms may have a more dynamic impact on bacterial autoaggregation than that of the carbohydrate sources utilized in the experiment.

CONCLUSIONS

Generally, neither prebiotic carbohydrates, monosaccharides nor disaccharides significantly impacted the autoaggregation in aggregative or non-aggregative lactobacilli strains. While the majority of the carbohydrate sources did not promote the autoaggregation of most lactobacilli strains, 2FL prompted the rapid autoaggregation of *Lactobacillus acidophilus* La-5. There exists abundant research concerning how probiotics function while few scientific studies account for how autoaggregation might be a factor in how probiotics inhibit pathogens from colonizing the intestinal systems. We investigated the extent to which carbohydrates were limitations to the abilities of lactobacilli strains to autoaggregate. The rapid stimulation of *Lactobacillus acidophilus* La-5 to autoaggregate by 2FL contributes to the necessary research investigating the mechanisms involved with probiotic bacteria and carbohydrate interactions. Further research is necessary to understand the interactions of 2FL with this strain. This finding may warrant further research in interactions between probiotic bacteria and carbohydrates, perhaps with additional strains. If autoaggregation is a crucial element of efficient probiotics, further research is needed to investigate what factors affect autoaggregation. The experimental results, overall, warranted scientific research that focused on carbohydrates as potential, influential factors on the autoaggregation of lactobacilli strains. Modified experiments need to focus on factors that impact autoaggregation of probiotic bacteria *in vivo*, aside from isolated carbohydrates.

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