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Full Length Research Paper

Relationship between intestinal bifidobacteria content and ABO antibody titer

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Intestinal biota may stimulate the development of anti-ABO antibodies. The goal of this study was to verify whether there is a relationship between fecal bifidobacteria concentration and anti-ABO titer in healthy subjects. This study recruited individuals with A, B, and O blood types for anti-ABO antibody titer at AT and through the HAG test. Fecal pH was verified and the concentration of fecal bifidobacteria was determined using a selective-medium quantitative culture. Among the 129 volunteers, the frequency of ABO phenotypes was 44.5% of Group A, 9% of Group B, and 46.5% of Group O. No relationship was observed between the bifidobacteria concentration and the anti-A and anti-B titer in AT and HAG. Between the ABO phenotypes, the one with the highest bifidobacteria concentration was Group A, followed by O and B. The differences fecal bifidobacteria concentration significantly differed between Groups A and B and between O and B, but not between A and O. The results suggest that the antigens present in the bifidobacteria seem not to impact immunomodulation in Group B individuals and that the anti-ABO titer is not related to this bacteria genus. More studies on may explain which bacteria are able to increase the ABO antibodies titer.

Key words: Antibody formation, *Bifidobacterium*, ABO blood group system.

INTRODUCTION

The antibodies against ABO antigens make this system the most important in blood transfusions since transfusing ABO-incompatible blood components incompatibility may lead to an intravascular hemolytic reaction and cause kidney failure or patient death (Roback et al., 2008; Stroka et al., 2009).

The development of ABO system antibodies occurs in a natural or immune way. The immune development occurs when the individual has contact with incompatible ABO erythrocyte antigens in blood transfusion, pregnancy, or miscarriage, which will cause anti-A, anti-B, and/or anti-AB alloantibodies to develop. This alloimmunity may also be developed through contact with anti-diphtheria or anti-tetanus serums of animal or bacterial origin (Roback et al., 2008; Girello and Kühn, 2011). An increase in antibody titers against ABO-system antigens has been shown in individuals who received pneumococcal vaccination containing A-like antigens (Boyer et al., 1981; Koskela et al., 1988). The lack of blood-group substances in the influenza virus vaccine led to a better vaccine which did not increase the ABO-system antibody titer in those who received the vaccine, and their blood components did not cause transfusion reactions (Delaney et al., 2011).

Natural ABO antibody immunization begins at around three to six months after birth and these antibodies' titer decrease after 65 years of age (Roback et al., 2008; Girello and Kühn, 2011). Bacteria that have antigens similar to those present in erythrocytes, such as *Escherichia coli* $O^{86}B7$, passively stimulate the formation

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Abbreviations: AT; Ambient temperature, **HAG**; Indirect human antiglobulin.

of these anti-ABO antibodies (Springer and Horton, 1969).

Some microorganisms use the blood-group carbohydrates in mucosae to bind to tissues (Stowellv et al., 2010; Uchida et al., 2006; Wu et al., 2006). For instance, *Helicobacter pylori* is adapted to use the ABO antigens in the stomach mucosa as a binding molecule, which enables its dissemination (Springer and Horton, 1969). Likewise, the genus *Lactobacillus* uses adhesins and collagen in the stomach mucosa to bind to the intestinal lumen and may also use ABO antigens as receptors and ligands (Uchida et al., 2006). It is also known that the ABO-system antigens in tissues are more complex than those in erythrocytes since the ones in the mucosa are greatly influenced by the Lewis (secretory) system (Wu et al., 2006).

In the intestinal biota, the bifidobacterium genus is the largest in the colon and makes up over 25% of the total gut flora in adults and 95% in newly-borns (Berg, 1996; Cummings and Macfarlane, 2002). Considered non-pathogenic bacteria, bifidobacteria play beneficial biological roles to human health and are used in probiotic products to restore intestinal biota in cases of imbalance (Cummings and Macfarlane, 2002; Bouhnik et al 2005).

In this sense, given the evidence of increased immune response in individuals who consume probiotics (Daniel-Johnson, et al., 2009), the natural bacterial diversity of the intestinal biota (Berg, 1996; Cummings and Macfarlane, 2002), and the scarcity of studies correlating the intestinal bacteria and the antibodies against AOB-system antigens, this study hypothesizes that the anti-ABO antibody titer of an individual is proportional to the amount of bacteria in the intestine, particularly bifidobacteria.

Thus, this study aimed to verify whether there is a relationship between fecal bifidobacteria concentration and anti-ABO antibody titer in healthy subjects.

MATERIALS AND METHODS

Study design

This research project was carried out according to the guidelines and norms of Resolution 196/96 of the National Health Board/Ministry of Health, of the Declaration of Helsinki (2000) by the World Health Association, and of Resolution 116/96 of Brazilian Health Surveillance Agency (ANVISA). The number of volunteers in the study was calculated considering the mean frequency of ABO-antibody elevated titers observed in blood donors in researches carried out in Brazil (Fernandes et al., 2008; Gambero et al., 2004). Healthy volunteers between 18 and 65 years old were randomly recruited in the cities of Blumenau and Florianópolis, SC, Brazil. Subjects with suspected or diagnosed autoimmune diseases or diabetes, who had consumed products containing prebiotics, symbiotics, and/or probiotics in the three months prior to the research, who had been vaccinated or had fever, diarrhea, or vomit in the three months prior to the research, who had been pregnant in the 30 months prior to the research, who had AB phenotype, and who had positive irregular antibody screen test results were excluded from the study.

Two blood samples were collected from the volunteers, one to determine the titer of serum antibody against ABO-system antigens and the other to determine the ABO phenotype and carry out plasma irregular antibody screen test. One feces sample was required to quantify the bifidobacteria and measure fecal pH.

The antibodies against ABO-system antigens were titrated at the Immunohematology Laboratory of the Regional Blood Bank of Blumenau (Center of Hematology and Hemotherapy of Santa Catarina (HEMOSC)). The bifidobacteria were quantified and fecal pH was analyzed at the Department of Clinical Analyses of the Federal University of Santa Catarina (UFSC).

ABO phenotyping and irregular antibody screen

The blood sample collected with EDTA was used for the ABO phenotyping and irregular antibody screen following the legislation in effect in Brazil (Brasil, 2013). The result of the ABO phenotyping was concluded by means of direct and reverse grouping using anti-A, anti-B, and anti-AB monoclonal serums and 3% reagent red blood cells from Bio-Rad Laboratories®. The *in vitro* technique followed the reaction's intensity reading pattern as described in the literature (Girello and Kühn, 2011).

The irregular antibody screen was performed *in vitro* using reagent red blood cells (DiaCell I+II®) and reaction potentiators (DiaLiss) from Bio-rad Laboratories®. The monospecific anti-human globulin IgG used *in vitro* was Coombs-Serum IgG® from Bio-Rad-Laboratories®. *In vitro* irregular antibody screen was performed by putting 50 µL reagent red blood cells into glass hemolysis tubes and adding 100 µL of the samples to each tube. After homogenization, the tubes were centrifugeg for 15 s at 3,400 rpm. The ambient temperature (AT) reaction was read using an aglutinoscope and 100 µL DiaLiss® were then added to each tube. The indirect anti-human globulin (AHG) step was carried out by incubating the tubes with DiaLiss® for 5 min at 37°C and then washing them three times with saline solution. Next, 100 µL AHG were added. The reaction was read using an aglutinoscope after 15 s of centrifugation at 3,400 rpm. The *in vitro* technique followed the reaction intensity reading pattern according to the literature. (Girello and Kühn, 2011) For the negative reactions in the AHG step, they were confirmed with Coombs Control red blood cells (Bio-Rad Laboratories®) according to the current legislation in Brazil (Brasil, 2013).

Determining anti-A and anti-B antibody titer

Anti-A and anti-B titers were determined according to the literature (Judd et al., 2008) using serial titration of the subjects' serum. Two *in vitro* serial titration batches down to 1/512 were carried out to determine the antibody class, one for AT and the other for AHG. The samples collected with no anticoagulant were centrifuged at 4,000 rpm for 5 min. Next, 100 µL serum were added to the first test tube and then serial dilution was performed down to 1/512. After that, 50 µL 3% A_1 red blood cell suspension (DiaCell ABO (A_1+B)®) from Bio-rad Laboratories® were added to each tube previously identified as HA and 50 µL of 3% B red blood cell suspension (DiaCell ABO (A_1+B)®) from Bio-rad Laboratories® were added to each tube previously identified as HB.

Ambient temperature (AT) step

In order to titrate the antibodies against IgM-class ABO-system antigens, the tubes were homogenized and centrifuged at 3,400 rpm for 15 s. The reaction was read to check for the presence or absence of hemolysis and/or agglutination. In case hemolysis and/or agglutination were verified in the 1/512 titer, the solution was further diluted.

Indirect human antiglobulin (AHG) step

For the titration of antibodies against IgG-class ABO-system antigens in AHG, the tubes were incubated in a water bath at 37°C for 60 min, however, no IgG immunoglobulin destruction technique was used. Next, the red blood cells were washed three times with a saline solution and 100 µL monospecific anti-human antiglobulin IgG (Coombs-Serum IgG®) from Bio-Rad Laboratories®. Next, the reactions were homogenized and centrifuged at 3.400 rpm for 15 min. The reaction was read to check for the presence or absence of hemolysis and/or agglutination. In case hemolysis and/or agglutination were verified in the 1/512 titer, the solution was further diluted.

Determining bifidobacteria content and fecal pH

The feces samples for isolation and quantification of bifidobacteria and fecal pH determination were analyzed within 8 h of collection.

From each volunteer, 1 g feces was weighed and diluted into 9 mL sterile distilled deionized water to verify the pH in a previously calibrated PHTEK® pH meter.

Another 1 g feces was also weighed and diluted into 9 mL 0.31 mM pH 7.2 phosphate buffer. From this dilution (10^{-1}), the serial decimal dilutions were performed by transferring 100 µL of the previous dilution into 900 µL phosphate buffer.

The RCA (Reinforced Clostridial Agar, Difco™ BD) culture medium supplemented with antibiotics (2% nalidixic acid, 0.85% polymyxin B sulfate, 0.5% kanamycin sulfate, 0.5% iodoacetic acid, 0.5% 2,3,5-triphenyltetrazolium chloride, and 0.001% amphotericin B) was used to isolate the bifidobacteria (Muñoa and PARES, 1988).

The seeding of 100 µL of each dilution was carried out in the RCA medium using a Drigalski spatula. The Petri dishes were incubated at 37°C in an anaerobiosis jar using a commercial anaerobic atmosphere generation system (Anaerobac from Probac®).

After 72 h of incubation, the bifidobacteria colonies were counted in the dishes containing between 30 and 300 CFU, observing the growth of different colony types. All colony types isolated underwent Gram staining, catalase assay, and fructose-6-phosphate-phosphoketolase (F6PPK) to confirm the genera (Scardovi, 1986; Orban and Patterson, 2000).

All rod- to coccoid-shaped Gram-positive and catalase-negative colonies were cultivated at 37°C for 48 h in 10 mL reinforced clostridial broth (Himedia) in anaerobiosis jars. After this period, the broths were centrifuged at 10,000 g at 4°C for 15 min. The pellets were washed twice with 0.05 M pH 6.5 KH_2PO_4 phosphate buffer containing 500 mg/L cysteine-HCL and then resuspended in 1 mL of the same solution. The bacterial cells were ruptured by adding 0.4 mL cetrimide bromide (45 mg/mL) for 5 min. Next, 0.25 mL of the 3 mg/mL NaF and 5 mg/mL sodium iodoacetate solution and 0.25 mL of the 80 mg/mL fructose-6-phosphate solution were added, mixed, and incubated at 37°C for 30 min. To halt the reaction, 1.5 mL 13 g/dL pH 6.5 hydroxylamine-HCL solution were added, mixed, and left at room temperature for 10 min. In order to acidify the medium, 15% 1 mL trichloroacetic acid (w/v) and 1 mL 4 M HCl were added. 1 mL of the ferric chloride color indicator (5% FeCl.6 H_2O in 0.1 M HCl) was added and the solution was stirred. The development of violet color indicated the presence of the enzyme F6PPK and the yellow color indicated a negative result (Orban and Patterson, 2000).

The bifidobacteria quantification results were presented as colony-forming units per gram of feces (CFU/g). For that end, the number of CFUs counted in the Petri dishes was multiplied by the respective dilution and corrected by the seeded sample volume.

Statistical analysis

The data was statistically analyzed using the software GraphPadPrism® version 5.0 (2007). For the data distribution analysis, D'Agostino-Pearson omnibus normality test was applied. Pearson test was used to verify the correlation between the bifidobacteria concentration, fecal pH, and titer of antibodies against ABO-system antigens. Spearman's correlation coefficient

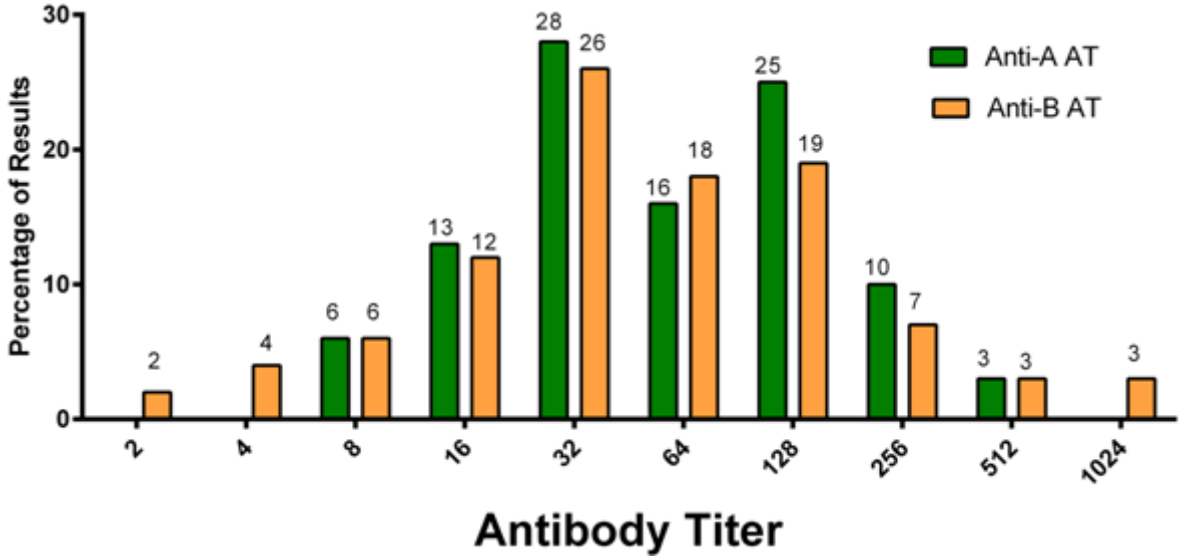

Figure 1. Percentage distribution of the results of titers of ABO-system antibodies with reaction reading at ambient temperature. The antibody titer is expressed only with the denominator. AT: Ambient Temperature.

was used to verify the correlation between the anti-ABO antibody titer and the volunteers' ages. Student's T-test was used to compare the fecal bifidobacteria concentration and fecal pH between different ABO phenotypes.

RESULTS

Initially, 158 volunteers were recruited and 157 blood samples were obtained. Among the 157 ABO phenotypings, 70 (44.6%) were classified as A, 14 (8.9%) as B, 67 (42.7%) as O, and 6 (3.8%) as AB.

Among the 158 individuals recruited, 29 were excluded from the study for the following reasons: one due to difficult venous access, 22 due to the exclusion criteria, and six for having AB phenotype. No subject had irregular antibodies.

Overall, 129 volunteers took part in the study and had the following breakdown of ABO phenotypes: 45% (58) group A, 9% (12) group B, and 46% (59) group O. Among the 129 volunteers, 93 (72.0%) were female and 36 (28.0%) were male. The mean volunteer age was 37±11 years (21 to 65 years). The mean interval between blood and feces sample collection was 4.1±6.4 days.

ABO-system antibody titer

The samples with anti-A antibodies at AT had titers between 1/4 and 1/512, with an average of 1/91±1/102 and the titer with the highest prevalence (trend) was 1/32. The titer for anti-B at AT ranged from 1/2 to 1/1024 with an average of 1/106±1/179 and the titer with the highest prevalence was also 1/32 (Figure 1).

The samples with anti-A antibodies in AHG had titers between 1/8 and 1/16384, with an average of 1/613±1/1981 and the titer with the highest prevalence was 1/128. The titer for anti-B in AHG ranged from 1/4 to 1/2048 with an average of 1/258±1/508 and the titer with the highest prevalence was also 1/128 (Figure 2).

No significant relationship was found between volunteer age and anti-A antibody titer at AT (p=0.8730) and in AHG (p=0.5603) (Figure 3). However, a significant relationship was found with anti-B antibody titer at AT (p=0.0075). No significant relationship was observed between anti-B antibody titer in AHG (p=0.1361).

Bifidobacteria and fecal pH

The bifidobacteria concentration in the volunteers' feces was, on average, $9.18\times10^{8}\pm55.2\times10^{8}$ CFU/g feces and the mean fecal pH was 7.03±0.59. Among the ABO phenotypes, the highest fecal bifidobacteria concentration was found in group A ($1.09\times10^{9}\pm5.58\times10^{9}$ CFU/g) and group B ($3.97\times10^{6}\pm11.7\times10^{6}$ CFU/g). The differences were significant between the fecal bifidobacteria concentrations of groups A and B (p<0.0001) and O and B (p<0.0001), but not between Groups A and O (p=0.4856).

No significant correlation was observed between the bifidobacteria concentration and fecal pH (p=0.2786). Among the ABO phenotypes, the highest fecal pH was found in group A (7.07±0.57), followed by group O (6.98±0.62) and group B (6.90±0.65). No significant difference was found in fecal pH among the ABO groups

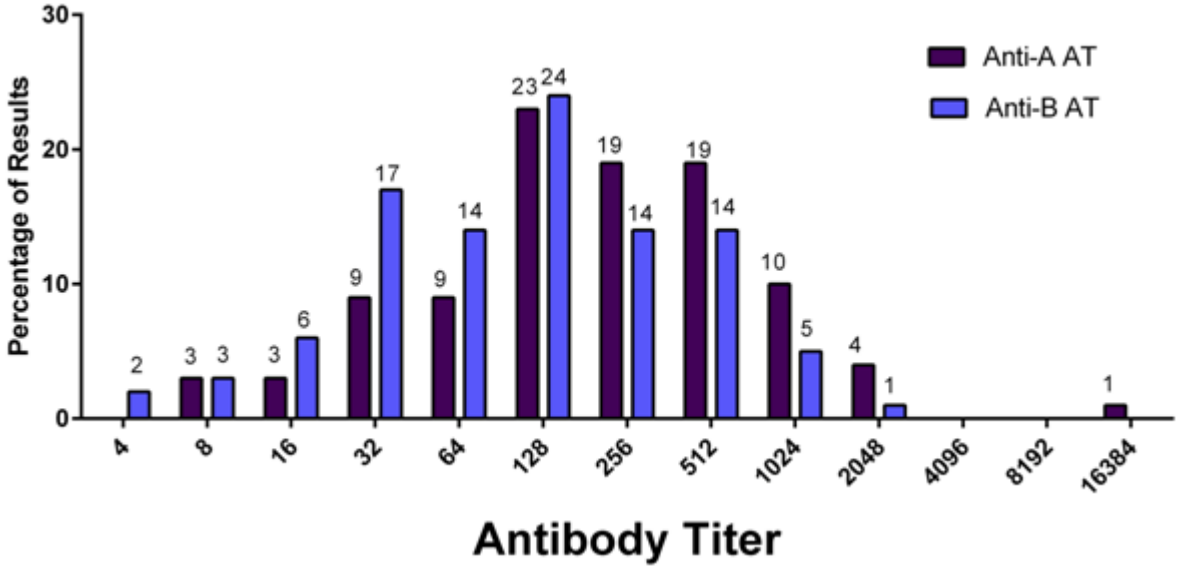

Figure 2. Percentage distribution of the results of titers of ABO-system antibodies with reaction reading in the anti-human globulin phase. The antibody titer is expressed only with the denominator. AHG: Anti-Human Globulin.

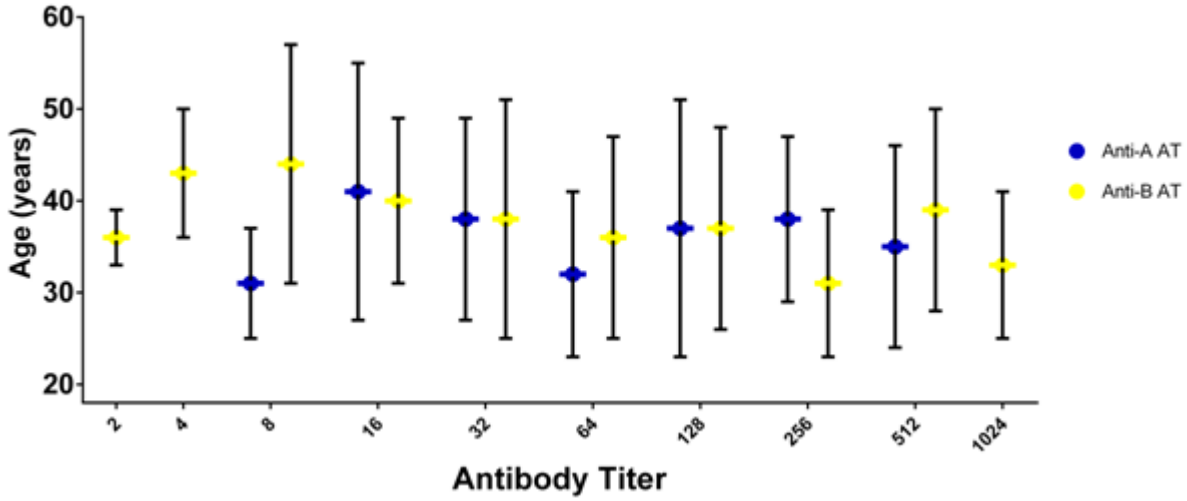

Figure 3. Anti-A and anti-B antibody titers observed with reading at Ambient Temperature according to age. AT: Ambient Temperature. The antibody titer is expressed only with the denominator. Age is expressed as mean±standard deviation.

(group A × O, p=0.5066; group A × B, p=0.5624; group O × B, p=0.8278).

No relationship was found between fecal bifidobacteria concentration and titer of antibodies against ABO-system antigens for the different ABO groups either. Likewise, no relationship was verified between bifidobacteria concentration and anti-A antibody titer at AT (p=0.5013) and in AHG (p=0.7888) and anti-B at AT (p=0.4878) and in AGH (p=0.6347) when all phenotypes were analyzed as a whole.

DISCUSSION

The frequency of ABO phenotypes, the prevalence of ABO antibody titers, as well as the variation in titers observed in the present study match the results found by other authors (Fernandes et al., 2008; Gambero et al., 2004). Using the *in vitro* technique, the most prevalent anti-A and anti-B titer at AT in group-O platelet donors in the USA was 1/16 and 1/8, respectively (Cooling et al., 2008). The same prevalences of anti-A and anti-B titers

(1/16) were reported in Japan and Thailand. In AHG, the prevalent titers of 1/8 and 1/32 for anti-A and 1/32 and 1/256 for anti-B were observed, respectively, in Japan and Thailand (Mazda et al., 2007). In Brazil (São Paulo), the most prevalent anti-A titer at AT was 1/64 among blood donors (Gambero et al., 2004), a similar result to the one in the present study. The preset results showed that the ranges of anti-ABO antibody titers at AT were higher than the ones found in other countries (Cooling et al., 2008; Mazda et al., 2007), but similar to those described in the Brazilian literature (Gambero et al., 2004). The greater variation in anti-A and anti-B antibody titers in AHG than at AT may be related to the presence of group-O samples since this ABO phenotype has IgG-class anti-AB antibodies, which are reactive with AHG (Roback et al., 2008; Girello and Kühn, 2011).

The changes in antibody titers among the Japanese between 1986 and 2005 was attributed to the culture change in the country, particularly to the higher consumption of Western (industrialized) products over these years (Mazda et al., 2007). Transfusion reactions caused by the transfusion of iso group non-ABO platelet components were attributed to a change in diet by a group-A blood donor, who began showing anti-B titers of 1/16,384 (Daniel-Johnson, et al., 2009). Among the 129 subjects, a group-O volunteer had anti-A antibody titer of 1/16,384. The prevalence of anti-A and anti-B antibody titers found in the present study was below those that caused hemolytic transfusion reactions due to minor ABO incompatibility (Cooling et al., 2008; Daniel-Johnson, et al., 2009).

A recent study showed a direct relation between ABO groups and intestinal biota. In that study, with approximately half the number of subjects of the present research, group O had the highest fecal bifidobacteria concentration and group B had the most *Lactobacillus* bacteria. The highest fecal bifidobacteria concentration was found in group-O subjects, followed by groups AB, B, and A. Nevertheless, those authors did not relate this concentration to the antibody titers (Mäkivuokko et al., 2012). The lack of a significant relation between the anti-ABO antibody titers and the fecal bifidobacteria concentration may be attributed to the different dietary habits of the current volunteers and, consequently, to the presence of different prevalent species in the intestinal biota of each subject. This is supported by the fact the different concentrations of bifidobacteria species have already been found among the ABO phenotypes (Mäkivuokko et al., 2012; Wacklin et al., 2011). The discrepancy may also be attributed to another blood system (Lewis), since the secretory subjects had higher fecal bifidobacteria concentrations compared to the non-secretory.

The transfusion reactions described by Daniel-Johnson (Daniel-Johnson, et al., 2009) did not point out the species and strains the blood donors had ingested prior to the donation of blood components containing high titers of antibodies against ABO-system antigens, but instead only specified the genera *Lactobacillus*, *Bifidobacterium*, and *Bacillus subtilis*. In the present study, no significant relation was observed between the bifidobacteria concentration and the titer of ABO antibodies in subjects who did not consume prebiotics, probiotics, or symbiotics, however, only the *Bifidobacterium* genus was analyzed. Another study also did not find a significant difference in the titers of anti-A and anti-B antibodies after the intake of probiotic milk by children, using the strains *Lactobacillus acidophilus* CRL730 and *Lactobacillus casei* CRL431 (Pérez et al., 2010). Those authors attributed the lack of effect of probiotics on immunoglobulins to the improvement in sanitation conditions in the country, which makes the children less exposed to intestinal infections. They also believe that this natural infection process is key to the development of the immune system (Pérez et al., 2010).

Nonetheless, the mere presence of the ABO monosaccharide in the bacteria cell wall is not enough to stimulate the development of ABO antibodies. Instead, it must be an active carbohydrate since only one of the monosaccharide enantiomers is immunogenic (Stowellv et al., 2010; Wu et al., 2006). Although studies report that bacteria may adhere to the ABO antigens (Stowellv et al., 2010; Uchida et al., 2006; Wu et al., 2006) and stimulate the increase in ABO-system antibody titers (Springer and Horton, 1969; Daniel-Johnson, et al., 2009), it is possible that other components of mucin such as the carbohydrate chains, sialic acid, and proteins are responsible for the intensity with which the bacteria adhere to the intestinal mucosa (Uchida et al., 2006). Moreover, natural lectins present in individuals of the A and B groups are able to kill *Lactobacillus acidophilus* and *Bifidobacterium lactis* (Stowellv et al., 2010; Uchida et al., 2006) and could regulate the intestinal biota in these volunteers.

Although pH may also aid in regulating the intestinal biota, the present research did not observe a significant relation between fecal pH and bifidobacteria concentration or differences in pH among the different ABO groups. However, fecal pH may not reflect colon pH precisely since, in order for intestinal metabolism to be reflected in feces, variables such as intestinal mobility, total fiber intake, and intestinal secretions such as short-chain fatty acid production must be considered (Gibson and Roberfroid, 1995; Blaut, 2002; Garro et al., 2005).

No relation between intestinal bifidobacteria and anti-ABO antibody titer was found, however, a significant difference was observed in the concentration of intestinal bifidobacteria among the different ABO phenotypes. The results suggest that the antigens present in the bifidobacteria seem not to impact immunomodulation in group-B individuals and that the anti-ABO antibody titer is not related to this bacteria genus. Despite the studies showing that dead *Escherichia coli O*^{86} may raise the

anti-ABO antibody titer (Springer and Horton, 1969), other intestinal bacteria could also impact these titers (Uchida et al., 2006; Mäkivuokko et al., 2012; Tabasum and Nayak, 2011; Wacklin et al., 2011). Hence, the use of foods and/or drugs able to modulate intestinal biota could directly impact the anti-ABO antibody titer and, consequently, change the frequency of individuals with high anti-ABO antibody titers.

Therefore, further studies investigating the relation between intestinal biota and anti-ABO antibody titer could explain the heterogeneity in the frequency of individuals with high ABO-system antibody titers in the same population or within the same ABO blood group.

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