**An Acad Bras Cienc (2021) 93(2): e20190989 DOI 10.1590/0001-3765202120190989**
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

# The effect of carbohydrates on the adherence of *Pasteurella multocida* to the nasal respiratory epithelium

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**Abstract:** *Pasteurella multocida* subsp. *multocida* is responsible for different diseases that generate great economic losses in farm animal. The effectiveness of immunization against those bacteria are variable and the use of antibiotics is questioned; for that reason, we investigated the potential inhibitory effect of different carbohydrates on the adherence in vivo of *P. multocida* to the rabbit respiratory epithelium as an alternative for the prevention of respiratory infections. Rabbits were intranasally and intratracheally inoculated with a solution containing 200 µl of $1x10^7$ CFU of *P. multocida* that was previously mixed with 250 µg /200 µl of N-acetylglucosamine, alphamethylglucoside, alphamethylmannoside, N-acetylgalactosamine or sialic acid. The animals that received N-acetylglucosamine, alphamethylglucoside or alphamethylmannoside individually or a mixture of these three carbohydrates plus the bacterium, showed a significant decrease ($P < 0.05$) of the clinical symptoms, microscopic and macroscopic lesions in the nasal septa and in the lungs; also, the number of adhered bacteria to the nasal epithelium were also significantly reduced. This research demonstrates for the first time that such an approach could convert into a method for prevention of *P. multocida* infection in rabbits that is ecologically and economically safe and effective.

**Key words:** Adherence, carbohydrates, lesions, *Pasteurella multocida*.

## INTRODUCTION

*Pasteurella multocida* is a Gram negative bacterium that normally resides in the uper respiratory tract of many animal species. Five capsular serotypes (A, B, D, E and F) and 16 somatic serotypes of the microorganism have been described (Frost & Adler 2000, Boyce & Adler 2006, Dziva et al. 2008, Dagleish et al. 2010, Hatfaludi et al. 2010) which are associated with specific diseases among different animal species. The respiratory complex of rabbits is more frequently associated with the serotypes A:3 and A:12 and less commonly with serotype F of *P. multocida* (DiGiacomo et al. 1990, Borkowska-Opacka et al. 1995, Kawamoto et al. 1997, Boyce et al. 2004, Dziva et al. 2008, Jaglic et al. 2008, Massacci et al. 2018).

The macroscopic, histological and ultrastrucutral changes in the nostril of rabbits suffering from natural disease or undergoing different experimental protocols with *P. multocida* have been thoroughly described. Macroscopically, congestion of the nasal mucosa, hemorrhage and mucopurulent exudate are reported (Al-Haddawi et al. 2000, 2001). The main histologic change is described as catarrhal suppurative rhinitis (Botero & Iregui 1999, Al-Haddawi et al. 2001); while the ultrastructural alterations consist of degeneration of epithelial cells, cytoplasmic vacuolization, loss of cilia, necrotic cells,

infiltration of polymorphonuclear heterophiles and macrophage between degenerated epithelial cells and in the subepithelial layer; finally, hyperplasia and hypertrophy of goblet cells are reported (Al-Haddawi et al. 2001, Esquinas et al. 2013). The pathogenesis of the disease caused by *P. multocida* in rabbits, and even in other more well investigated species has been poorly studied. The estimated prevalence of the infection by this microorganism is between 7% and 100% in healthy rabbits (Dziva et al. 2008, Massacci et al. 2018). Rabbits often are colonized with *P. multocida* for long periods of time without showing clinical signs. Infection is often acquired from a carrier dam, and the disease develops when the animals are subjected to some form of stress like transportation, overcrowding, or changes in the temperature or humidity of their environment, which appear to favor an exaggerated microbial proliferation and virulence due to mechanisms that are not completely understood (Jordan & Roe 2004, Dziva et al. 2008, Jaglic et al. 2008). The economic losses due to *P. multocida* in rabbits are high and its control has been difficult, similar consequences are reported with the respective *P. multocida* in a wide variety of domestic animal species under intensive production conditions (Dziva et al. 2004, Dagleish et al. 2010).

*P. multocida* produces many diverse virulence factors including a capsule composed of highly hydrated polyanionic polysaccharides which are covalently bound to the surface of bacteria through phospholipids or lipid A; outer membrane proteins (OMP), which may serve as adhesins or participate in the formation of biofilms as type IV fimbrae; the fibronectin binding protein and filamentous hemagglutinin (Hatfaludi et al. 2010).

In an *in vitro* model of HeLa cell monolayer and pharyngeal parakeratotic cells cultures, Glorioso et al. (1982) achieved the inhibition of the adhesion to the two cell types by N-acetyl-D-glucosamine of *P. multocida* A obtained from rabbits, this led the authors to suggest that there are lectin-like molecules on the bacterial surface, specifically in the fimbriae, that would act as carbohydrate binding ligands with the NacGlu configuration on both host epithelial surfaces (Ruffolo et al. 1997); this role has been specifically attributed to fimbria type IV of this bacterium (Hatfaludi et al. 2010, Jacques 1996).

Cellular vaccines are used to control infections by *P. multocida*, some of which are made with inactivated bacteria but they may present problems due to reactivation, probably due to the content of endotoxin in any case, they do not produce long term immunity (Ataei et al. 2009, Shivachandra et al. 2014). Differences have been found between antibody response and protection for bacteria such as *Pasteurella* spp. It is possible that total IgG antibodies induced by rPmOmpA do not contain opsonic antibodies, which would decrease the phagocytic capacity of neutrophils (Dabo et al. 2008). On the other hand, while a cellular immune response occurs as a result of infections by *P. multocida*, the bacterium can subvert that response such that it induces apoptosis in immunocompetent cells (Praveena et al. 2010). Some have maintained that the immune response against *P. multocida* is mainly humoral through the antibody response to LPS; however, despite the availability of both live and killed vaccines for prevention of this infection, few offer good protection given the diversity of LPS in this microorganism (Harper et al. 2013).

On the other hand, the resistance of *P. multocida* to antibiotics is well known (Dowling et al. 2004). Resistance has been demonstrated in this organism against penicillin, chloramphenicol, tetracyclines, aminoglycosides and streptomycin (Katsuda et al. 2013, Jamali et al. 2014).

Given that the surface of the respiratory mucosa of the rabbit, as in other species, is rich in glycoproteins (Kooyk & Rabinovich 2008), as well as the observation that some of the adhesins of *P. multocida* have lectin-like properties with the capacity to bind to carbohydrates, we began this study in an attempt to inhibit the adhesion of the bacterium to the respiratory epithelium of rabbits using different carbohydrates. N-acetylglucosamine, alpha methyl glucoside and alpha methyl mannoside demonstrated the ability to prevent the onset of clinical disease and the lesions caused by this organism. Even more, a mixture of these three carbohydrates showed more significant inhibition of the lesions when compared with each carbohydrate alone.

## MATERIALS AND METHODS

### *Pasteurella multocida* strain

*P. multocida* A strain AUN001 was obtained from samples of the nasal turbinates, trachea and lungs of rabbits with signs of rhinitis and pneumonia from farms of the Sabana de Bogota, Colombia. The microorganism was cultivated on brain-heart infusion (BHI) agar with 5% sheep's blood, and morphologically round, gray, non-hemolytic colonies were selected. Gram stain identified the bacteria as Gram negative coccobacilli which exhibited bipolar staining, and biochemically were catalase and oxidase positive. They did not grow on MacConkey agar, were not hemolytic, and were indole positive, urease negative, ornithine decarboxylase positive, glucose positive, lactose negative, sucrose positive, maltose negative, and mannitol positive (Dziva et al. 2008). For molecular characterization, the *hyaD* gene sequence of the *cap* locus was amplified using the primers: F. 5´TGC AAA AAT CGC AGT CAG 3´ R. 5´TTG CCA TCA TTG TCA GTG 3´.

### Carbohydrates

To assay the inhibition of adherence of *P. multocida* to the respiratory epithelium of the nasal cavity of rabbits, 5 sugars were used (Vector laboratories®): N acetylglucosamine (GlcNAc), alpha methylglucoside (AmeGlc), alpha methylmannoside (AmeMan), N acetylgalactosamine (GalNAc) and sialic acid (Neu5AC) in agreement with adhesion inhibition experiments previously carried out with lectins (Carrillo et al. 2015).

### Protocol of infection with *P. multocida* and adherence inhibition assays

All of the procedures were approved and authorized by the bioethics committee of the Faculty of Veterinary Medicine and Animal Husbandry of the Universidad Nacional de Colombia (Acta 006/2010).

Forthytwo clinically healthy, 35 day old New Zealand White rabbits that were microbiologically negative for *Bordetella bronchiseptica* and *P. multocida* were used. The animals were adapted for life in the animal facility for 15 days, and at 51 days of age were distributed randomly into 14 treatment groups, three rabbits per group (Table I).

200 µl of *P. multocida* at a concentration of $1x10^7$ colony forming units (CFU) in physiological saline were mixed with 250µg/200µl of physiological saline of each carbohydrate: GlcNAc (0,0056 M), AmeGlc (0,0064 M), AmeMan (0.0064 M), GalNAc (0,0056 M) y Neu5AC (0,0040 M) or with the mixture of carbohydrates deemed significant, for 15 minutes at room temperature. Each experimental group was instilled with each treatment solution intranasally (200 µL IN) and intratracheally (200 µL IT) as shown in Table I.

The animals were examined every four hours beginning at the time of instillation, and were evaluated for the presentation of clinical signs of respiratory infection and/or septicemia.

**Table I. Experimental mixtures of *P. multocida* with each one of the sugars and mixture of sugars.**

| Rabbit Group (n=3, each) | Experimental Solution |
|---|---|
| 1 | *P. multocida* + GlcNAc |
| 2 | *P. multocida* + AmeGlc |
| 3 | *P. multocida* + AmeMan |
| 4 | *P. multocida* + GalNAc |
| 5 | *P. multocida* + Neu5AC |
| 6 | GlcNAc |
| 7 | AmeGlc |
| 8 | AmeMan |
| 9 | GalNAc |
| 10 | Neu5AC |
| 11 | *P. multocida* + Mixture of GlcNAc + AmeGlc + AmeMan |
| 12 | Mixture of GlcNAc + AmeGlc + AmeMan |
| 13 | *P. multocida* (Positive Control) |
| 14 | SSF (Negative Control) |

For administration of the experimental solution as well as for euthanasia the animals were previously anesthetized with acepromazine at 0.5mg/kg via subcutaneous injection (SCT), xilazine at 5mg/kg via intramuscular injection (IM) and ketamine at 35mg/kg via IM. At 72 hours post-instillation the rabbits were euthanized with Euthanex® at a dose of 1mL/5Kg via intracardiac delivery.

## Tissue processing

After the death of the animals, the heads were removed and two cross sections of the nasal septum were made ahead of the first premolar, approximately 0.5 cm thick. The rib cage was removed and the complete respiratory system from the larynx was separated out and infused with 3.7% formalin delivered through the trachea with a column of 20 cm of water pressure to obtain complete fixation and major expansion of the lungs. Afterwards, sections of all the pulmonary lobes were made. The samples of the nasal turbinates and complete lungs were kept in formalin for 24 hours at 4°C. The sections of the nasal septum were later decalcified in a 10% solution of disodium EDTA, pH 7 for 8 days at 4°C. All of the tissues were processed by routine methods and stained with hematoxylin-eosin (H&E).

## Microbiological reisolation

Samples of the cranial lobe of the lung were taken for microbiological reisolation. The samples were cultivated on BHI agar at 37°C for 24 hours and characterized by Gram stain and the oxidase and catalase tests.

## Gross evaluation of the lungs

All lungs were macroscopically evaluated and cataloged according to the distribution of the lesions in: apparently normal lung or without evident anatomic/pathologic changes (AN); diffuse lesions characterized by generalized congestion, increased size of the lungs and marking of the costal arches (D); or cranial lesions characterized by red consolidation of the cranioventral region of the lung (CR).

## Microscopic evaluation of the tissues

Blind evaluation of the H&E stained tissues were carried out by the investigator using a light microscope with a 40X objective.

For the nasal septum, 6 fields in total on either side were evaluated: two from the dorsal region, two from the central region and two from the ventral region. The presence of neutrophil infiltrates into the epithelium was evaluated as well as increase of the interepithelial spaces and the presence of bacteria on the ciliate border.

To each of the changes a score was assigned according to the degree of severity ranging from the absence of any change to severe (Table II). For each nasal septum the scores for each change in each one of the fields were summed and an average rating calculated for each lesion.

In the lung, the sections of each lobe were evaluated, all were processed and a score assigned according to the degree of severity of the following lesions: thickening of alveolar septa, accumulation of detritus in the alveolar and/or bronchiolar lumen, focal pneumonia and presence of bacteria (Table II). For each lung the scores for each one of the lobes were added up and an average score for each lesion was calculated.

### Indirect immunoperoxidase

An indirect immunoperoxidase (IIP) technique was developed in order to stain *P. multocida* and determine its location over the tissues of those animals treated with a mixture of sugars. As primary antibody a sheep polyclonal antiserum against *P. multocida* as a 1:1 dilution, and for secondary antibody donkey anti-sheep IgG (Sigma, Aldrich®) were used at a dilution of 1:500. Chromogenic detection of peroxidase employed a commercial development kit (Liquid DAB Substrate Kit, Invitrogen™). Nasal septum and lung tissues were each assigned a ranking according to the epithelial area labeled with adherent bacteria that ranged from absent to severe (Table II).

**Table II. Ranking according to the degree of severity of each change or lesion in the nasal septum or lung and the percentage of area labeling of *P. multocida* by IIP over the epithelium of the nasal septum or lung.**

| Degree of the lesion/area of the staining of *P. multocida* by IIP | Assigned ranking |
|---|---|
| Absence/ 0% | 0 |
| Slight/ >0-33% | 1 |
| Moderate/ >33-66% | 2 |
| Severe/ >66% | 3 |

### Statistical analysis

The severity of lesions in the lung and nasal cavity, and the degree of labeling of *P. multocida* through IIP were rated from 0 to 3. The average scores for each group were evaluated via ANOVA to determine whether there were differences between treatments, which was followed by Dunnett's test to compare different treatments to the positive control. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

### The administration of individual sugars attenuates the presentation of clinical signs, reisolation of bacteria and macroscopic lesions caused by *P. multocida*

None of the animals exposed to *P. multocida* plus GlcNAc (group 1) and AmeGlc (group 2) presented clinical signs; while the rabbits treated with *P. multocida* and AmeMan (group 3), GalNAc (group 4) or Neu5AC (group 5) did have clinical signs. The predominant signs were fever, cyanosis of the mucosa and ears, dyspnea and mucopurulent nasal secretions. Animals in the positive control group (group 13) showed a greater severity of clinical signs. As well, none of the animals in the carbohydrate control groups (groups 6-10), nor in the negative control group (group 14) manifested evident signs. Figure 1 shows the number of animals that had clinical signs in the experimental groups exposed to *P. multocida* with sugars (groups 1-5) or only to *P. multocida* (group 13).

The presence of *P. multocida* was only detected in the lungs of those animals of groups 4, 5 and 13 by microbiological isolation. The rabbits treated with *P. multocida* + GlcNAc

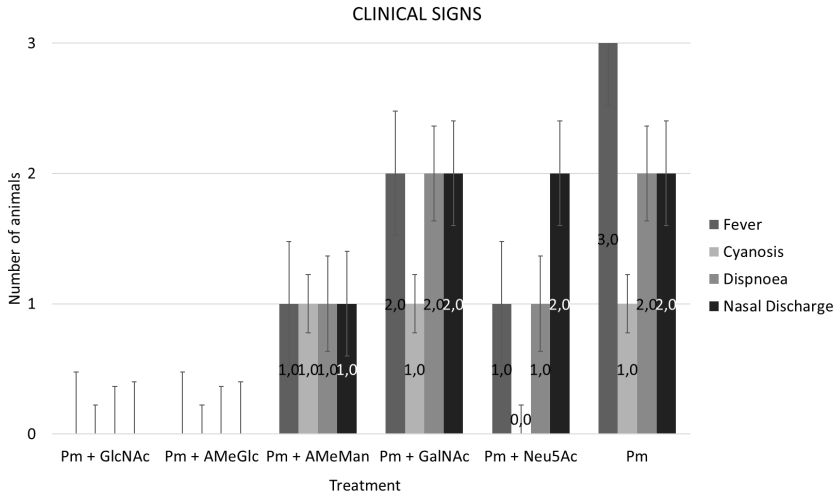


**Figure 1. Number of rabbits that showed clinical signs in each experimental group exposed to *P. multocida* with the corresponding sugars (groups 1-5) or to *P. multocida* alone (group 13). *P. multocida* (Pm), N-acetylglucosamine (GlcNAc), alphamethylglucoside (AMeGlc), alphamethylmannoside (AMeMan), N-acetygalactpsamine (GaINAc) or sialic acid (Neu5Ac).**

(group 1), *P. multocida* + AmeGlc (Group 2) and *P. multocida* + GlcNAc (Group 4) showed lungs that were apparently normal, and only one animal treated with *P. multocida* + AmeMan (group 3) presented symptom and a pattern of cranioventral bronchopneumonia. In the positive control group (13) and other groups that were inoculated with bacteria and other sugars (5), at least two rabbits showed some type of pulmonary lesion. None of the negative control animals (group 14), nor carbohydrate controls (groups 6-10) evidenced pulmonary injury (Figure 2 and Supplementary Material - Figure S3).

## The administration of individual sugar decreases the severity of microscopic lesions in nasal sept and lungs

### *Nasal septa*

In the rabbits of groups 1 (*P. multocida* + GlcNAc), 2 (*P. multocida* + AmeGlc) and 3 (*P. multocida* + AmeMan) the nasal septa were less significantly affected ($P<0.05$) in comparison to the microscopic lesions of the positive control group (13) (Figure 3). There were no microscopic changes in the negative control group (14) or the carbohydrate controls (groups 6-10).

Figures 4a and 4b correspond to rabbit nasal septa of groups 14 (negative control) and 1 (*P. multocida* + GlcNAc) respectively, which show normal architecture. In contrast, in the tissues corresponding to groups 4 (*P. multocida* + GalNAc) (Figure 4c) and 13 (positive control) (Figure 4d), there was evidence of the presence of bacteria, increase in interepithelial spaces and infiltration of PMNs into the epithelium.

### *Lungs*

In the lungs of animals of groups 1 (*P. multocida* + GlcNAc), 2 (*P. multocida* + AmeGlc) and 3 (*P. multocida* + AmeMan) the severity of all of the lesions was less ($p<0.05$) in comparison to the positive control (13) (Figure 5). There was no evidence of microscopic changes in the negative control (group 14) or in the carbohydrate controls (groups 6-10) (Figure 5).

Figure 6a shows a lung of a negative control animal (group 14) that conserves the normal architecture. Figure 6b corresponds to a rabbit lung of the positive control group (group 13) which shows a severe thickening of septa. Figure 6c shows a rabbit lung from group 1 (*P. multocida* + GlcNAc) very similar to the morphology of the lung in the negative control group. Figure 6d shows a lung from group 4, the observed changes are similar to those in the panel of positive control.

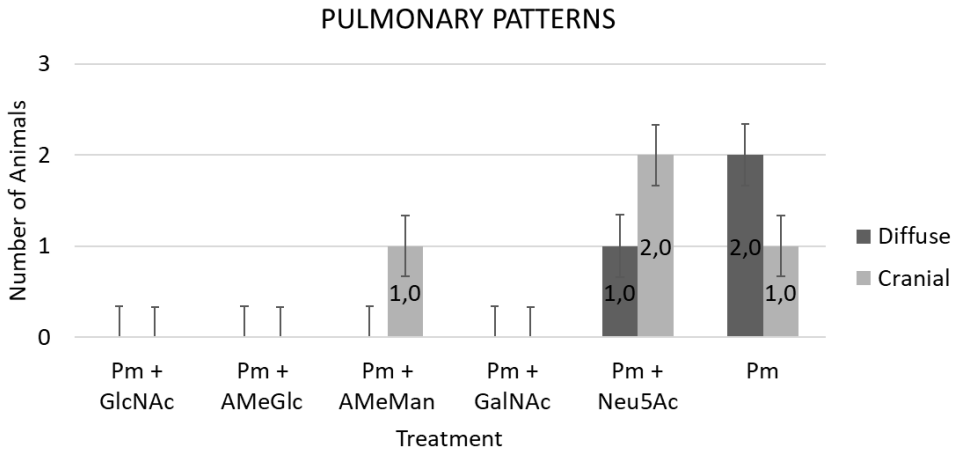


**Figure 2. Number of animals that manifested different patterns of pneumonia in each treatment group. *P. multocida* (Pm), N-acetylglucosamine (GlcNAc), alphamethylglucoside (AMeGlc), alphamethylamannoside (AMeMan), N-acetygalactosamine (GalNAc) or sialic acid (Neu5Ac).**

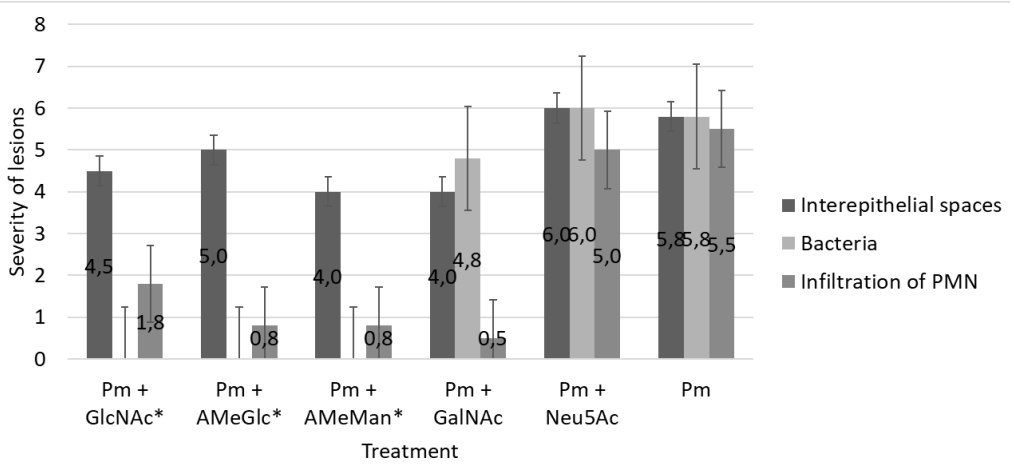


**Figure 3. Degree of severity of microscopic changes in the respiratory epithelium of the rabbit nasal septa in groups exposed to *P. multocida* + individual carbohydrates or to *P. multocida* alone (*P<0.05). *P. multocida* (Pm), N-acetylglucosamine (GlcNAc), alphamethylglucoside (AMeGlc), alphamethylmannoside (AMeMan), N-acetylgalactosamine (GalNAc) or sialic acid (Neu5Ac).**

## The mixture of sugar increase the inhibitory effect against adherence of *P. multocida*

A 1:1:1 mixture of GlcNAc, AmeGlc and AmeMan was used that represented the carbohydrates that better inhibited the manifestation of clinical signs, macroscopic lesions and significantly prevented the development of microscopic lesions in the rabbit nasal septa and lungs as well as significantly reducing the reisolation of *P. multocida*.

None of the animals exposed to *P. multocida* plus the carbohydrate mixture (group 11) or to the carbohydrate mixture without bacteria (group 12) manifested clinical signs.

At necropsy all of the rabbit lungs of group 11 (*P. multocida* plus carbohydrate mixture) and of group 12 (carbohydrate mixture alone) appeared normal.

Moreover, the animal group treated with *P. multocida* + the carbohydrate mixture (group 11) had lesions of a degree of severity that was significantly less ($p<0.05$) in nasal septa and lungs in comparison to the rabbits treated with *P. multocida* plus individual sugars that had significantly inhibited lesions by

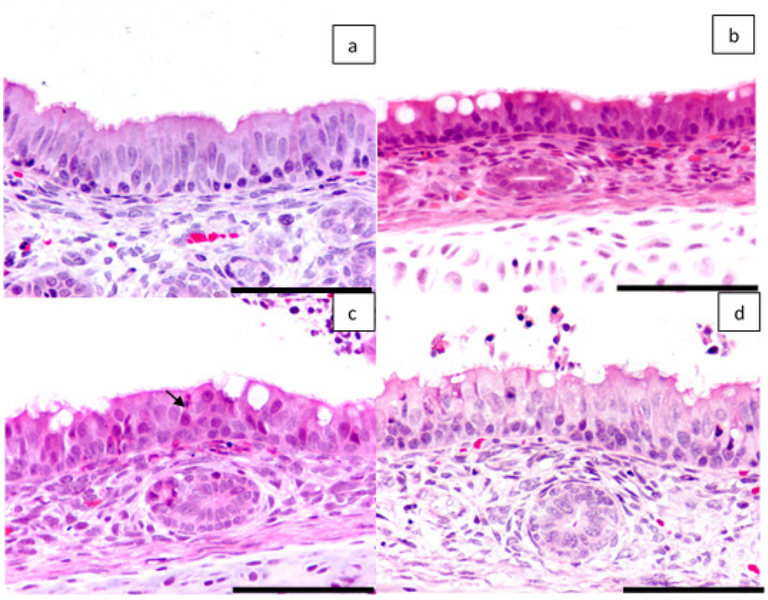


**Figure 4. a. Respiratory epithelium of rabbit nasal septum, negative control (group 14). Normal architecture of ciliated epithelium. H&E, 40X. b. Rabbit nasal septum treated with *P. multocida* + GlcNAc (group 1). The architecture is similar to that seen in Fig. 5a; the number of goblet cells is variable in normal animals. H&E, 40X. c. Ciliated epithelium of the rabbit nasal septum, positive control (group 13). The infiltration of at least 2 or 3 PMNs between the epithelial cells is evident (arrow) as well as the disorganization of the epithelial architecture (dysplasia); some inflammatory detritus accumulates in the lumen. H&E, 40X. d. Rabbit nasal septum treated with *P. multocida* + GalNAc (group 4). Disorganization of the epithelial architecture due to loss and necrosis of epithelial cells is evident. Desquamating epithelial death cells into the lumen. H&E, 40X. Scale bar: 200 µm.**

themselves (groups 1, 2 and 3) (Figures 7 and 8). The respiratory epithelium of the nasal septa of rabbits treated with *P. multocida* plus the mixture of carbohydrates (group 11) appeared normal (Figures 7 and 8).

The rabbit nasal septa and lungs (not shown) of group 13 (positive control) were immunoreactive to anti-*P. multocida* and positively stained by IIP (Figure S1), while those tissues in group 11 animals (*P. multocida* + carbohydrate mixture) were not reactive (Figure S2) ($P<0.05$).

## DISCUSSION

In general, infections by *P. multocida* are associated with high rates of morbidity and mortality in diverse species of mammals and birds (Dziva et al. 2004, Dagleish et al. 2010). Prevention, treatment and control of these infections is difficult due to the high resistance of these bacteria to antibiotics and the poor efficacy of vaccines (Dowling et al. 2004, Praveena et al. 2010, Harper et al. 2013, Katsuda et al. 2013). Therefore, the development of new strategies and tools for prevention and therapy are of the most importance in the management of these diseases. Among the strategies, the prevention of bacterial adherence to the apical surface of the respiratory epithelial cells of the host, the first step in the establishment of the infection by *P. multocida* and a moment of major vulnerability of the microorganism, appears to be a logical objective. This study demonstrated that the sugars GlcNAc, AmeGlc and AmeMan when used individually significantly inhibited the adherence of *P. multocida* to the respiratory epithelium of rabbits and thereby diminished

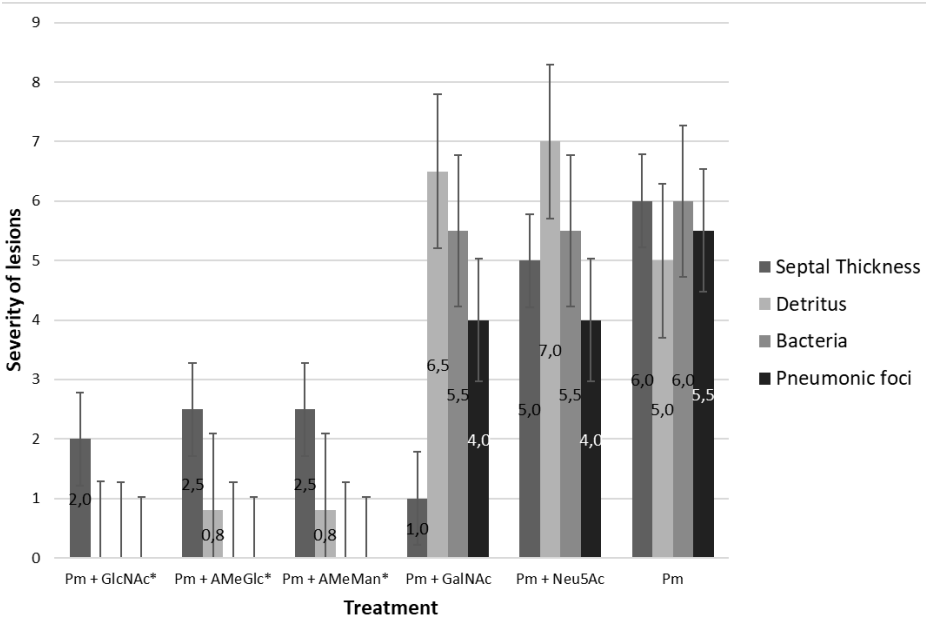


**Figure 5. Degree of severity of microscopic lesions in rabbit lung parenchyma in the groups treated with *P. multocida* + individual carbohydrates and with *P. multocida* alone (*P<0.05). *P. multocida* (Pm), N-acetylglucosamine (GlcNAc), alphamethyglucoside (AMeGlc), alphamethylmannoside (AMeMan), N-acetylgalactosamine (GalNAc) or sialic acid (Neu5Ac).**

the presentation and intensity of clinical signs, and macroscopic and microscopic lesions in the nasal septa and lungs of rabbits. Moreover, we demonstrated that a mixture of these 3 sugars significantly inhibited the adhesion of *P. multocida* and the clinical consequences and lesions caused by this pathogen in rabbits in comparison not only to the positive control but also when compared to the sugars administered individually.

In principle sugars do not attack the integrity of these microorganisms as happens with antibiotics and also with vaccines they are not even harmful to the host. Thus the genome of the microorganism is not subjected to selection pressure to evolve to a new, more virulent form in order to survive, and the host maintains its state of health and normal respiratory tissue. In sum, the use of sugars establishes a more organic relationship between the two. In this manner not only are the lesions produced at the cellular level by the microorganism prevented, but also those produced by the inflammatory response with deleterious consequences for the host (Tetley 1993, Mogensen 2009).

Glorioso et al. (1982) studied the adhesion of *P. multocida* A isolated from rabbits to cultured monolayers of HeLa cells and to parakeratotic cells of the pharynx. The most significant result was the inhibition of adhesion of the bacteria to both types of cells mediated by GlcNAc. This suggested that lectin-like molecules existed over the surface of the bacteria, specifically in the fimbrae, that acted as ligands for the binding of carbohydrates configured as GlcNAc over both epithelial surfaces of the host (Ruffolo et al. 1997).

The genome sequence analysis of *P. multocida* 3 Pm70 identified two genes for filamentous hemagglutinin (*fha*B1 y *fha*B2) for which similar pro-adherent activities to the filamentous hemagglutinin of *Bordetella pertussis* (FhaB-FhaB1, FhaB2) were proposed (Hatfaludi et al. 2010). At least three different

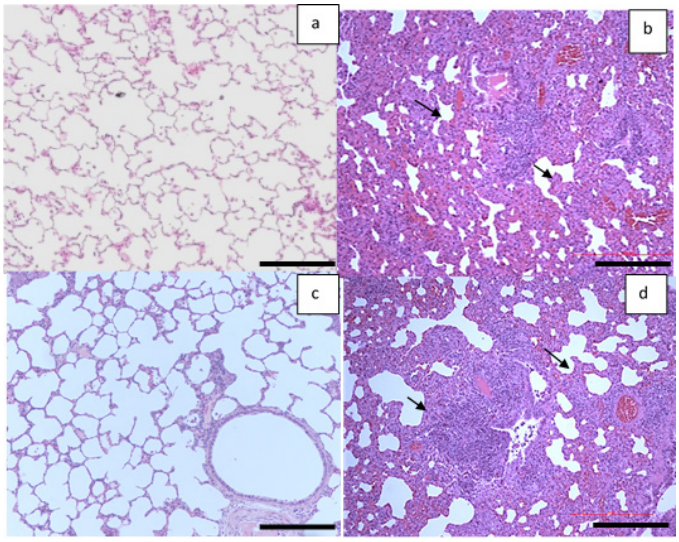


**Figure 6. a. Rabbit lung, negative control (group 14). The septa and the alveoli conserve the normal morphology of the organ without thickening or detritus, respectively. H&E, 10X. b. Rabbit lung, positive control (group 13). Severe thickening of the alveolar septa is evident due to the presence of inflammatory infiltrates; multifocal congestion is also evident. H&E, 10X. c. Rabbit lung treated with *P. multocida* and GlcNAc (group 1). There is only one focus of slight thickening of the alveolar septa (arrow). H&E, 10X. d. Rabbit lung treated with *P. multocida* + GalNAc (group 4). The observed changes are similar to those in the panel of positive control (7b). Severe thickening of the alveolar septa is evident due to the presence of inflammatory infiltrates, as well as multifocal congestion. H&E, 10X. Scale bar: 200 µm.**

binding site motifs for the filamentous hemagglutinin of *B. pertussis* have been described: one glucosaminoglycan that mediates binding to heparin, heparin sulfate and other sulfated carbohydrates; one arginine-glycine-aspartate sequence that mediates binding to leukocytes; and one carbohydrate domain that mediates adherence to ciliated respiratory epithelial cells and to macrophages (Tuomanen et al. 1988, Relman et al. 1990, Prasad et al. 1993, Hannah et al. 1994). The proteins FhaB1 and FhaB2 of *P. multocida* could have the same affinity for carbohydrate receptors of the respiratory epithelium of rabbits and would be candidate targets for the sugars used in this work.

Recent studies from our group carried out in a search for alternative strategies for the control of infections by *P. multocida* A demonstrated, employing an *ex vivo* model using the nasal septa of fetal rabbits, that the use of lectins with affinity for the carbohydrates D-Man, D-Glc, and GlcNAc significantly inhibited the quantity of bacteria adhering to the apical surface of epithelial cells and the activity of goblet cells (Carrillo et al. 2015). We therefore proposed that these sugars could diminish the appearance of lesions and clinical signs induced by this pathogen, as indeed verified in this study.

Similar results to those documented here have been described by *in vitro* studies with *Pseudomonas aeruginosa* in which it was demonstrated that sugars such as heparin, dextran and dextran sulfate inhibit the adherence of that microorganism to epithelial cells of the A549 line from the respiratory tract (Bavington & Page 2005). Findings of the same nature were obtained for *Burkholderia cenocepacia* and *Legionella pneumophila* using mono-, di- and trisaccharides like GalNAc _ 1–4Gal, GalNAc _ 1–3Gal, Gal _ 1–4GlcNAc and Gal _ 1–3GlcNAc (Bavington & Page 2005).

The intratracheal administration in rabbits of oligosaccharides like lacto-*N*-neotetraose (LNnT) and their α 2–3– and α 2–6–sialylated derivatives, GalNAc b1–3 Gal, or GalNAc b1–4 Gal attenuated the course of pneumonia by *Streptococcus pneumoniae* when applied before exposure to the bacteria and prevented the colonization of the nasopharynx by that pathogen. In addition to that it drastically diminished the colonization of the lung and protected against bacteremia. Moreover, the same carbohydrates used in a therapeutic manner diminished the

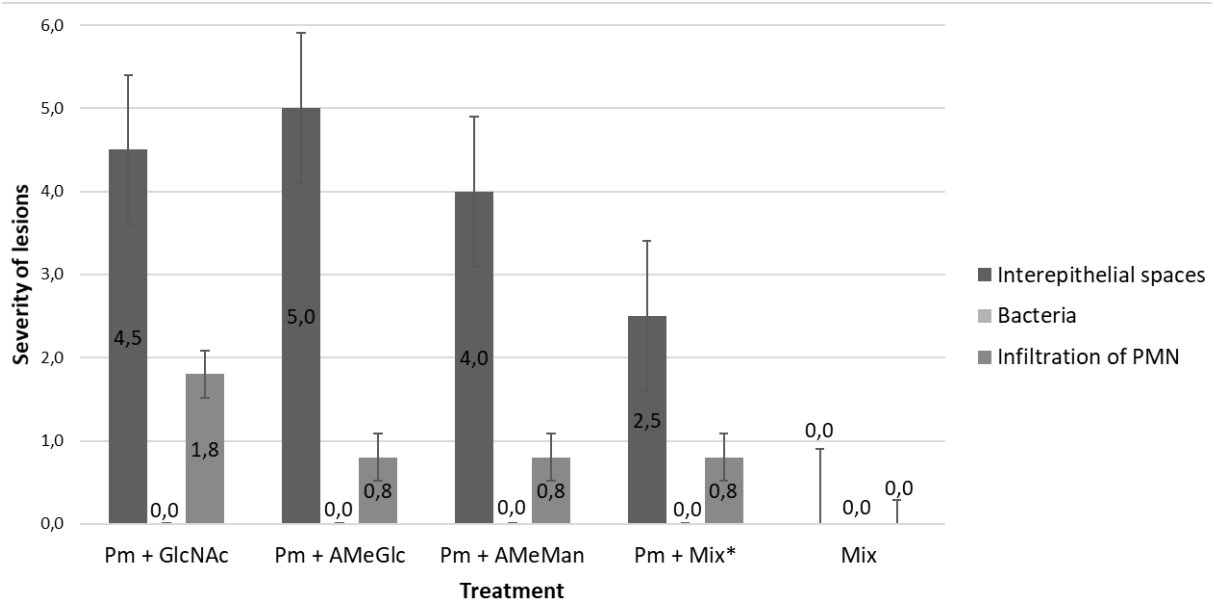


**Figure 7. Comparison of the severity of microscopic lesions in nasal septa among animals treated with *P. multocida* + individual carbohydrates and animals treated with *P. multocida* + the mixture of carbohydrates (*P<0.05). Mix= mixture of carbohydrates. *P. multocida* (Pm), N-acetyglucosamine (GIcNAc), alphamethyglucoside (AMeGIc), alphamethyglucoside (AMeGIc), alphamethylmannoside (AMeMan).**

intensity of the pneumonia and bacteremia when they were administered 24 hours after the infection was established. Administered intranasally, these neoglycoconjugates equally prevented the colonization of the nasopharynx by *Streptococcus pneumoniae* in infant rats. The partial correlation between the bioactivity *in vivo* and the inhibition of the adherence *in vitro* suggests that the oligosaccharides reduced the disease by *S. pneumoniae*, at least in part, by interfering with the adherence of the bacteria to the cells of the host. In this role, the oligosaccharides presumably act like soluble homologous receptors that bind to the bacteria inhibiting their subsequent adherence to the cells of the host (Idänpään-Heikkilä et al. 1997).

As a result of our work with carbohydrates, we formulated the hypothesis that by preventing the adhesion of *P. multocida* to the surface of the respiratory epithelium of rabbits, the animals would eliminate the bacteria from the respiratory tract through the non-destructive mechanism of the mucociliary escalator. Consistent with this Idänpään-Heikkilä et al. (1997) proposed that it is possible that the oligosaccharides used by them favored the elimination of pneumococci. They suggested that the oligosaccharides persisted in high concentrations in the thin film of the air-epithelial interface and therefore blocked the adherence of the bacteria even many hours after its application.

In this study, the mixture of sugars GlcNAc, AmeGlc and AmeMan had at least an additive effect - which was statistically significant in comparison to the individual carbohydrates - in the inhibition of the adhesion of *P. multocida* to the respiratory epithelium of the rabbits, as well as in the disminution of clinical signs and macroscopic and microscopic lesions. The inhibitory effect of the carbohydrate mixture found in this work derived from the block of major diverse adhesion sites of the

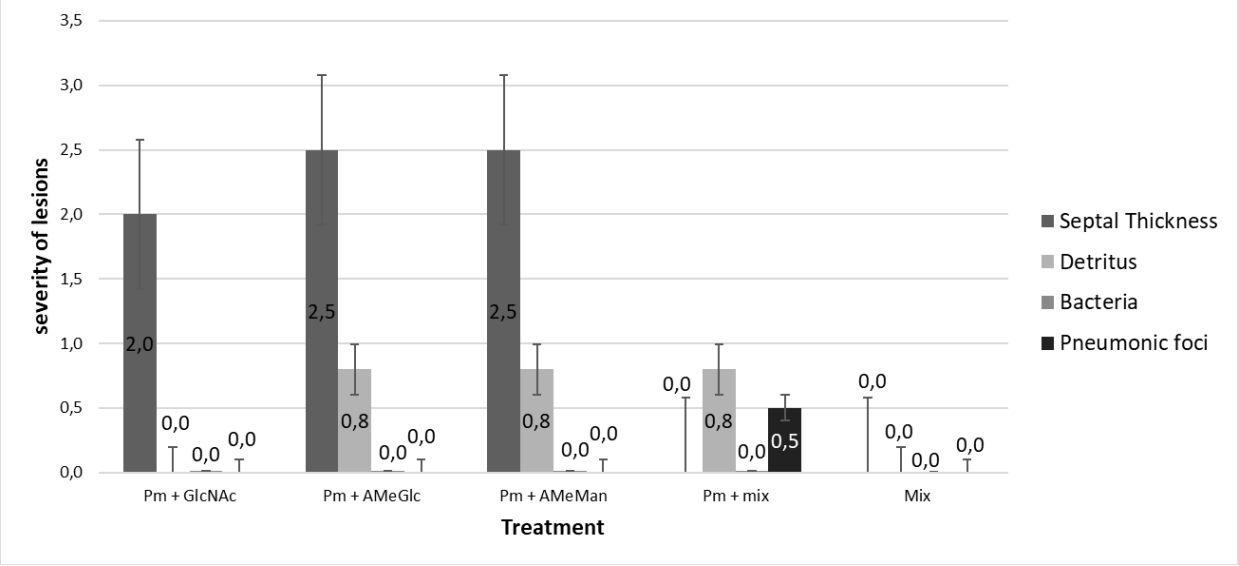


**Figure 8. Comparison of the severity of microscopic lesions in lungs among animals treated with *P. multocida* plus individual carbohydrates and animals treated with *P. multocida* plus the mixture of carbohydrates (P<0.05). Mix= mixture of carbohydrates. *P. multocida* (Pm), N-acetylglucosamine (GlcNAc), alphamethylglucoside (AMeGlc), alphamethylmannoside (AMeMan).**

bacteria that in turn indirectly point to the existence of different adhesion structures with different compositions on the surface of the microorganism. Other researchers support the hypothesis that some microorganisms have multivalent lectin-type ligands and therefore to achieve an effect of inhibition of adhesion, they must be blocked by various sugars, as is the case of Lec A and Lec B lectins of *Pseudomonas aeruginosa* that are blocked by preincubation with galactose and fucose; these concentrated solutions of carbohydrates are called glycoclusters, glycopolymers or glycodendrimer. Some research has shown that glycocclusters are successful in controlling infections by *Streptococcus suis*, uropathogenic *E. coli* and HIV through the use of glycodendrimers as anti-adhesion (Johansson et al. 2008, Audfray et al. 2013, Sattin & Bernardi 2016).

The effect of the carbohydrates are not limited only to a preventive activity. We should add that therapies with substances like the carbohydrates studied here, in addition to their obvious ecological and economic advantages, would have other beneficial properties, to mention only one of these: the low possibility that microorganisms would eventually develop resistance against them (Sharon 2006, Kulkarni et al. 2010, Sattin & Bernardi 2016).

In conclusion, the results of this investigation demonstrated that the previous incubation of *P. multocida* with individual GlcNAc, AmeGlc and AmeMan inhibited significantly the adherence of the bacterium to the respiratory epithelium of rabbits. Equally so, it prevented the expression of clinical signs, and microscopic and macroscopic changes in the nasal septa and lungs of rabbits experimentally exposed to the microorganisms with individual sugars; moreover, a mixture of these three carbohydrates showed, at least, a summatory inhibitory effect. This approach could convert into a method of prevention and treatment for *P. multocida* infections in rabbits that is ecologically and economically safe and effective. It is possible that the nasal and intratracheal instillation of these sugars has the

same preventive effect as mixing bacteria with the carbohydrates prior to instillation. Further studies should point to the development of more stable glycoconjugates with more prolonged effects and direct application to the animals, in addition to assessing their direct preventive activity against the pathogen, and as well to the exploration of a possible therapeutic effect against infection by *P. multocida.*

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## SUPPLEMENTARY MATERIAL

**Figure S1. Rabbit nasal septum of positive control (group 13). Positive IIP reaction indicates the presence of *P. multocida* antigen on the ciliated border and in the cytoplasm of the goblet cells of the nasal septa-IIP (arrows), 100X. Scale bar: 50 µm.**
**Figure S2. Nasal septum treated with *P. multocida* + the mixture of carbohydrates. Absence of any labeling that indicates the presence of *P. multocida*. IIP, 100x. Scale bar: 50 µm.**
**Figure S3. Lungs of experimental rabbits. Shows the lungs of a rabbit of the positive control group (group 11) presenting a cranial pneumonic pattern. b. Lungs of experimental rabbits. shows lungs with a normal appearance from a rabbit treated with *P. multocida* + GlcNAc (group 1).**

**How to cite**
GALLEGO C, PATIÑO P, MARTÍNEZ N & IREGUI C. 2021. The effect of carbohydrates on the adherence of *Pasteurella multocida* to the nasal respiratory epithelium. An Acad Bras Cienc 93: 20190989. DOI 10.1590/0001-3765202120190989.

*Manuscript received on August 23, 2019; accepted for publication on May 4, 2020*

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## Author contributions

Gallego and Iregui conceived and designed the study; Gallego and Patiño conducted the field work and laboratory experiments; Martínez analyzed the data; Gallego, Patiño and Iregui wrote the manuscript, all the authors read and approved the final version.