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MANNANOLIGOSACCHARIDE AGGLUTINATION BY *SALMONELLA ENTERICA* STRAINS ISOLATED FROM CARRIER PIGS

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ABSTRACT

Type-1 fimbriae are associated with most *Salmonella enterica* serovars and are an essential factor for host colonization. Mannanoligosaccharides (MOS), a prebiotic that is agglutinated by type-1 fimbriae, are proposed for the control of enterobacteria colonization and may be an alternative to *Salmonella* control in pigs. The aim of this study was to evaluate the capability of porcine *Salmonella* strains to adhere to MOS *in vitro*. A total of 108 strains of *Salmonella* sp. isolated from carrier pigs were evaluated for the amplification of *fim*A and *fim*H genes, agglutination of MOS and hemagglutination. In all tested strains, amplicons of expected size were detected for both *fim*A and *fim*H gene. In the hemagglutination assays, 31 (28.7%) strains presented mannose–sensitive agglutination of erythrocytes, indicating that the strains were expressing type-1 fimbriae. Considering only strains expressing the type-1 fimbriae, 23 (74.2%) presented a strong agglutination of MOS, 3 (9.6%) a weak reaction and 5 (16.2%) none. The results indicate that *Salmonella enterica* strains expressing type-1 fimbriae can agglutinate effectively *in vitro* to MOS.

Key words: *Salmonella*, type-1 fimbriae. mannanoligosaccharides

INTRODUCTION

Salmonella sp. has been frequently isolated from pigs at slaughter in Southern Brazil (4, 6), demonstrating the need of control measures implementation to reduce the risk of public health problems arising from the consumption of contaminated pork. *Salmonella* control can be implemented at the pre-harvest level (on farm), at harvest level (during transport and slaughter) and at post-harvest level (processing and retailing). When a high *Salmonella* prevalence on farm is detected, the first measures must be initiated at pre-harvest level to achieve a lower number of carrier pigs at slaughter

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and decrease the hazard of carcass contamination (10). Besides the measures related to animal management practices, the interest about alternative methods to inhibit the transmission of pathogens in farm animals has increased (5). Among these alternative methods, the use of prebiotics has been proposed to decrease the *Salmonella* infection in swine and poultry (12, 21).

Mannanoligosaccharides (MOS) are complex carbohydrates, derived from the cell wall of *Sacharomyces cerevisiae,* with mannose receptors that can bind to receptor sites of pathogenic bacteria, avoiding the attachment to the epithelium. Specifically, MOS is a prebiotic that has the ability to adhere to type1 fimbriae and has been used as an alternative to antibiotic use for growth promotion and therapeutic treatment in the swine industry (18).

Fimbriae play a critical role in the colonization by facilitating the initial attachment to specific host cells and tissues (3, 9, 15). Type-1 fimbriae are associated to most *Salmonella enterica* serovars and are characterized by their ability to mediate the mannose-sensitive agglutination of red blood cells (1, 25). Interactions between type-1 fimbriae and D-mannose-containing receptors have been shown in a number of studies to play a key role in the infectious process (1, 2, 8, 9, 17).

Many gene clusters corresponding to fimbrial systems are present in the genomes of *S. enterica,* however, only the *fim* cluster that code for type-1 fimbriae is clearly associated to the colonization of the gut. In the genus *Salmonella,* type-1 fimbriae are composed primarily of FimA proteins subunits, encoded by the *fim*A gene. However, another protein subunit called FimH has been shown to represent the fimbrial lectin, indispensable for the attachment to the gut epithelium (7, 11, 16, 20).

A study (21) conducted with strains of four *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Choleraesuis and *S.* Pullorum) demonstrated that the ability to adhere to MOS *in vitro* can vary between different serovars and among strains of a same serovar. In the same study, it was observed that the ability of MOS agglutination *in vitro* correlated well with the efficacy of MOS to decrease the *Salmonella* caecal counts in artificially inoculated chicks, demonstrating that feed supplemented with MOS may constitute an intervention measure in *Salmonella* control programs. Few studies have been conducted in pigs concerning *Salmonella* adhesion and colonization in the presence of prebiotics (12, 26). Moreover, the capability of MOS agglutination by *Salmonella* serovars commonly isolated from pigs has not been tested.

Thus, the aim of this study was to determine the capability of agglutination to mannanoligosaccharides in porcine *Salmonella enterica* strains expressing type-1 fimbriae.

MATERIAL AND METHODS

Strains: One hundred and eight *Salmonella enterica* strains comprising 26 serovars, isolated from carrier pigs were evaluated. The strains were isolated from feces, lymph nodes and tonsils of slaughtered pigs in southern Brazil. After identification and serotyping, strains were stored in Brain Heart Infusion with glycerol (20% v/v) at -20°C.

Isolation of DNA from bacteria: For preparation of the genomic DNA for multiplex PCR assays, overnight cultures in BHI at 37°C were prepared. One milliliter was subjected to DNA isolation using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

Primers for the Multiplex PCR: For the detection of *fim*A gene the primers fimAF 5'- CCT TTC TCC ATC GTC CTG AA-3'; fimAR 5'- TGG TGT TAT CTG CCT GAC CA-3', described by (7) were used, resulting in a 84 bp

amplicon. For the detection of *fim*H gene, primers were designed based on the sequences available (Genbank access number L19338). The primer sequence was selected by using the program Primer3 (www.primer tool, University of Massachusetts Medical School, U.S.A). The forward primer fimHF: 5'- ATG AGC ATC ACC GAT AGT GT-3' and the reverse primer fimHR 5'-GAA ATC AAA CTC CAC GAC CT-3', amplified a region of 311 bp between nucleotides 322 and 633 of the *fim*H gene of *S*. Typhimurium.

PCR: The PCR was carried out in a 25 µl mixture consisting of 50mMTris/HCl (pH 8.3), 200 µM (each) dATP, dCTP, dGTP and dTTP, 0.5 µM (each) primer, 0.65 U of Taq DNA polymerase (Invitrogen), 50 mM $MgCl_2$ and 2.0µL genomic DNA. The amplification was achieved on a thermocycler (Applied Biosystems, GemAmp PCR System 9700) as follows: an initial denaturation cycle at 94 °C for 5 min, followed by 25 cycles of 1 min at 94 °C, 30 s at 56 °C and 1 min at 72 °C, and a final extension at 72 °C for 7 min. Amplification products were separated by electrophoresis on 1.2% agarose gel and fragments were revealed with Blue Green Loading Dye I followed by visualization under UV. *Salmonella Typhimurium* (LT2) was used as a positive control in each PCR run. A template control (sterile water) was included to monitor contamination of the PCR reagents in each PCR assay.

Specificity of the multiplex PCR: DNA from non-*Salmonella* strains (*E. coli*, *Proteus vulgaris* ATCC 10145, *Klebsiella pneumoniae* ATCC13883, *Citrobacter freundii, Serratia marcescens* ATCC13889) was isolated and submitted to PCR amplification as described above.

Hemagglutination: Strains were submitted to hemagglutination test, using guinea pig erythrocytes with and without 1% mannose addition. Bacteria were grown overnight on Clumping Factor Agar (CFA, 25) at 37°C. The suspensions were adjusted to McFarland standard #4 in PBS or PBS/mannose 1% and serially diluted from 1:2 to 1:128 in the same buffer. Erythrocytes 1% (in PBS or PBS/mannose 1%) were added to each bacteria dilution. Suspensions of bacteria and erythrocyte were incubated overnight at 4ºC. After the incubation, suspensions were evaluated for the presence or absence of agglutination. All assays were conducted in triplicate. As positive control for the assays, an *Escherichia coli* strain (Type-1 fimbriae positive) was used. A suspension of erythrocytes 1% in PBS or PBS/mannose was running in parallel as the negative control. *Salmonella* strains that presented mannose-sensitive hemagglutination were considered expressing type-1 fimbriae as previously proposed (1, 13).

Slide agglutination assays of MOS: The MOS preparation used in this study was the commercial product BioMos® (Alltech, Kentucky, USA). For the agglutination assays of MOS, adjusted suspensions of *Salmonella*, as described above, were mixed with equal volumes of MOS 0.1% in PBS and kept at 4ºC for 1 hour. Aliquots of these suspensions were disposed on a glass slide and the agglutination of MOS was observed with a light microscope. *Salmonella* agglutination of MOS was compared with the result obtained in assays with the *E. coli* control strain and the clumping level was scored as follow: *strong*, when the same level of clumping was observed for tested and control strains; *weak*, when the clumping level observed for the *Salmonella* strain was lower than the control strain; *none*, when no clumping was observed. All assays were conducted in triplicate. A suspension of MOS 0.1% in PBS kept at 4ºC for 1 hour was used as negative control.

Statistical analysis: Results of mannose sensitive-hemmaglutination and agglutination of MOS were analyzed using the MacNemar test (Graphpad Instat 3.06) with

significance level P<0.05.

RESULTS AND DISCUSSION

One hundred and eight *Salmonella* strains isolated from carrier pigs submitted to multiplex PCR, targeting *fim*A and *fim*H gene, amplified fragments of the expected size (Figure 1). Strains from other related bacteria tested in the same assay were all negative, demonstrating that the assay is specific. Cohen *et al*. (7) proposed the primer set for the amplification of *fim*A gene and were able to detect 376 strains of *Salmonella* comprising over 80 serovars. The primers demonstrated a high specificity and were proposed as target to the detection of *Salmonella* in food samples. Similarly in our study, the gene *fim*A was detected in porcine strains of 27 *Salmonella* serovars, including S. Rissen, S. Gold Coast, S. Newport, S. Lexington e S. Grupensis, which were not tested in the previous report.

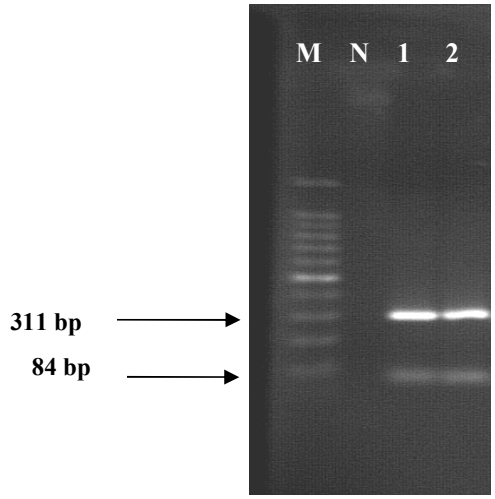

Figure 1. PCR multiplex amplicons (311bp *fim*H e 84 bp *fim*A) in 1.2% agarose: 100 pb molecular weight (M); negative control (N); *Salmonella* Typhimurium (1); *Salmonella* Bredeney (2).

For the purpose of our study, it was also important to detect the gene *fim*H that codes for the lectin, responsible for adhesion to the mannose receptor on the cell surface. The same mechanism is proposed for the agglutination of MOS by *Salmonella*, resulting in decrease of intestinal adherence (11, 19, 21). Thus, the detection of both genes in all tested porcine *Salmonella* strains indicated the potential capability to agglutinate MOS.

In the type-1 fimbriae expression assays, 31 strains (28.7%) presented mannose–sensitive agglutination of erythrocytes, and were considered as expressing type-1 fimbriae. In the assays using MOS, 30 strains (27.8%) showed a strong agglutination, 24 (22.2%) a weak reaction, and 54 (50.0%) none (Table 1). Considering only strains expressing the type-1 fimbriae, 23 (74.2%) presented a strong agglutination of MOS, 3 (9.6%) a weak reaction and 5 (16.2%) none. Among strains negative for mannose-sensitive hemagglutination, 49 (63.7%) were also negative on assays with MOS, 21 (27.3%) presented a weak reaction and 7 (9%) presented a strong agglutination. A good agreement between mannose-sensitive hemagglutination reaction and agglutination of MOS on the slide assay was observed (P<0.0001), demonstrating that *Salmonella enterica* strains expressing type-1 fimbriae *in vitro* can effectively agglutinate MOS.

No difference on the ability of MOS agglutination was found between strains isolated from feces and lymph nodes. Among strains isolated from feces (n=42), 47.6% agglutinated MOS, while 48.5% of the strains isolated from lymph nodes and tonsils (n=66) showed positive results.

Although multiple adhesins have been described for *Salmonella* Typhimurium, the type-1 fimbriae is the only one which have been shown to contribute to the colonization of the porcine intestinal tract (1, 2, 3, 5). In spite of that, a

Table 1. Agglutination with mannanoligosaccharide (MOS) and hemmaglutination mannose sensitive in *Salmonella enterica* strains isolated from pigs.

Serogroup	Serovar	Number of isolates	MOS agglutination			Hemmaglutination mannose sensitive	
			Strong	Weak	None	Positive	Negative
B	Agona	2	1	1		2	
	Brandenburg	1			1		1
	Bredeney	6	4		2	3	3
	Derby	4		1	3		4
	Heidelberg	3		1	2	1	2
	Typhimurium	38	10	9	19	10	28
C1	Infantis	3		2	1	1	2
	Mbandaka	3	1		2	1	2
	Montevideo	4		1	3		4
	Ohio	1		1			1
	Rissen	4	1	1	2	1	3
	Tennessee	5	1	1	3	2	3
	Gold Coast	3		2	1		3
	Newport	4			4		4
D1	Enteritidis	2			2		2
	Panama	2	1		1	1	1
E1	Anatum	3	3				3
	Give	3	2	1		2	1
	London	2	2			2	
	Orion	3	1	1	1	2	1
E4	Senftenberg	3	1		2	1	2
F4	Lexington	1			1		1
G2	Grupensis	2			2		2
	Havana	1			1		1
K	Cerro	3	2	1		1	2
L	Minessota	2		1	1	1	1
Total		**108**	**30**	**24**	**54**	**31**	**77**

switch to a nonagglutinating phenotype can occur at a rate of 10^{-2} to 10^{-5} per generation of *Salmonella* (1), and it has been demonstrated that some *Salmonella* strains with the *fim*A sequence could present a nonfimbriated phenotype *in vitro* (14, 22). The expression of type-1 fimbriae has been related to signals like temperature, osmolarity and bile salts concentration, which correspond to the environment present in the intestine (22). Recently, the on-off-phase variable expression pattern that has been proposed for the type-1 fimbriae of *Salmonella* (23, 24) was associated to global regulator proteins in addition to *fim*-specific proteins. This regulation form is likely to make the *fim* gene cluster sensitive to environmental stimuli and to physiological state of the cell (1, 3). Thus, the low frequency of *Salmonella* strains expressing type-1 fimbriae in our study can be related to any one of these factors, since the tested strains were stored at low temperatures and cultivated in artificial media prior testing. However, the widespread presence of the gene

*fim*A and *fim*H among the porcine *Salmonella* strains indicate the potential of expressing type-1 fimbriae and agglutinating MOS. Further investigation on potential of this feed additive to control *Salmonella* infection need to be conducted in pigs inoculated with strains expressing type-1 fimbriae.

CONCLUSION

The results indicated that MOS can be effectively agglutinated by porcine *Salmonella enterica* strains expressing type-1 fimbriae, and has potential to be tested to control the colonization of the intestine by *Salmonella* strains.

RESUMO

Aglutinação de cepas de *Salmonella enterica* isoladas de suínos portadores ao mananoligossacarídeo

Fímbrias tipo 1 estão presentes na maioria dos sorovares de *Salmonella enterica* e são fatores essenciais para a colonização do hospedeiro. Mananoligossacarídeo (MOS), um prebiótico que aglutina com fímbria tipo 1, tem sido proposto para o controle da colonização de enterobactérias e pode ser uma alternativa para o controle da infecção por *Salmonella* sp. em suínos. O objetivo desse estudo foi avaliar a capacidade *in vitro* de aglutinação ao MOS em cepas de *Salmonella* sp. isoladas de suínos. Um total de 108 cepas de *Salmonella* sp. foram avaliadas quanto à presença dos genes *fim*A e *fim*H, aglutinação ao MOS e hemaglutinação. Em todas as cepas testadas, fragmentos de tamanho esperado foram amplificados para ambos os genes. Nos testes de hemaglutinação, 31 (28,7%) cepas apresentaram aglutinação de hemácias inibida pela manose, indicando que havia expressão de fímbria tipo 1. Considerando apenas as cepas com a expressão de fímbria tipo 1, 23 (74,2%) apresentaram uma aglutinação forte ao MOS, 3 (9,6%) uma reação fraca e 5 (16,2%) não aglutinaram. Os resultados indicam que MOS pode aglutinar *in vitro* de forma efetiva com cepas de *Salmonella enterica* que estejam expressando fímbria tipo 1.

Palavras-chave: *Salmonella*, suíno, mananoligossacarídeo

REFERENCES

1. Althouse, C.; Patterson, S.; Fedorka-Cray, P.; Isaacson, R. (2003). Type 1 fimbriae of *Salmonella enterica* serovar Typhimurium bind to enterocytes and contribute to colonization of swine in vivo. *Infect. Immun*., 71: 6446-6452.
2. Bäumler, A.J.; Tsolis, R.M.; Heffron, F. (1996). Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. *Infect. Immun*., 64: 1862-1865.
3. Bäumler, A.J.; Tsolis, R.M.; Heffron, F. (1997). Fimbrial adhesins of *Salmonella typhimurium*. Role in bacterial interactions with epithelial cells. *Adv. Exp. Med. Biol.,* 412:149–158.
4. Bessa, M.C.; Costa, M.; Cardoso, M. (2004). Prevalência de *Salmonella* sp. em suínos abatidos em frigoríficos sob inspeção federal no Rio Grande do Sul. *Braz. J. Vet. Res*., 24: 80-84.
5. Boyen, F.; Haesebrouck, F.; Mae, D.; Van Immerseel, F.; Ducatelle, R.; Pasmans, F. (2008). Non-typhoidal Salmonella infection in pigs: A closer look at epidemiology, pathogenesis and control. *Vet. Microbiol*., 130: 1-19.
6. Castagna, S.M.F., Schwarz, P., Canal, C.W., Cardoso, M. (2004). Prevalência de suínos portadores de *Salmonella* sp. ao abate e contaminação de embutidos tipo frescal. *Acta Sci. Vete.*, v.32, p.141-147.
7. Cohen H.J.; Mechanda, S.M.; Lin, W. (1996). PCR amplification of the fimA gene sequence of *Salmonella* Typhimurium, a specific method for detection of *Salmonella* sp. *Appl. Environ. Microbiol.*, 62: 4303-4308.
8. Firon, N.; Ofek, I.; Sharon, N. (1982). Interaction of mannose-containing oligosaccharides with the fimbrial lectin of *Escherichia coli*. *Biochem. Biophys. Res. Commun*., 105:1426–1432.

9. Gahring, L.C.; Heffron, F.; Finlay, B.B.; Falkow, S. (1990). Invasion and replication of *Salmonella typhimurium* in animal cells. *Infect. Immun*., 58: 443-448.
10. Hurd, H.S.; Enoe, C.; Sorensen, L.; Wachman, H.; Corns, S.M.; Bryden, K.M.; Greneier, M. (2008). Risk-based analysis of the Danish pork Salmonella program: past and future. *Risk Analysis*, 28 (2): 341-350.
11. Kisiela, D.; Sapeta, A.; Kuczkowski, M.; Stefaniak, T.; Wieliczko, A.; Ugorrski, M. (2005). Characterization of FimH adhesions expressed by Salmonella enterica Serovar gallinarum biovar gallinarum and pullorum. Reconstitution of mannose-binding properties by single amino acid substitution. *Infect. Immun*., 73: 6187-6190.
12. Letellier, A.; Messier, S.; Lessard, L.; Chenier, S.; Quessy, S. (2001). Host response to various treatments to reduce Salmonella infections in swine. *Can. J. Vet. Res*., 65: 168-172.
13. McFarland, K.A.; Lucchini, S.; Hinton, J.C.D.; Dorman, C.J. (2008). The leucine-responsive regulatory protein, Lrp, activates transcription of the fim operon in *Salmonella enterica* serovar Typhimurium via the fimZ regulatory gene. *J. Bacteriol*., 190: 602-612.
14. Mirelmann, D.; Altman, G.; Eshdat, Y. (1980). Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeast. *J. Clin. Microbiol*. 11: 328-331.
15. Muller, K.H.; Collinson, S.K.; Trust, T.J.; Kay, W.W.J. (1991). Type 1 fimbriae of *Salmonella enteritidis*. *J. Bacteriol*., 173(15):4765-72.
16. Naravaneni, R.; Jamil, K. (2005). Rapid detection of food-borne pathogens by using molecular techniques. *J. Med. Microbiol.*, 54, 51–54.
17. Naughton, P.J.; Grant, G.; Bardocz, S.; Allen-Vercoe, E.; Woodward, M.J.; Pusztai, A. (2001). Expression of type 1 fimbriae of SEF *Salmonella enterica* serotype Enteritidis in the early colonisation of the rat intestine. *J. Med. Microbiol.,* 50:191–197.
18. Newman, K.E.; Newman, M.C. (2001). Evaluation of mannanoligosaccharide on the microflora and immunoglobulin status of sows and piglet performance. *J. Anim. Sci.,* 79: 189-191.
19. Oyofo, B.A.; DeLoach, J.R.; Corrier, D.E.; Norman, J.O.; Ziprin, R.L.; Mollenhauer, H.H. (1989). Effect of carbohydrates on *Salmonella typhimurium* colonization in broiler chickens. *Avian Dis*., 33: 531-534.
20. Sokurenko, E.V.; Courtney, H.S.; Ohman, D.E.; Klemm, P.; Hastyl, D.L. (1994). FimH family of Type 1 fimbrial adhesins: Functional heterogeneity due to minor sequence variations among fimH genes. *J. Bacteriol.*, 176: 748-755.
21. Spring, P.; Wenk, C.; Dawson, K.A.; Newman, K.E. (2000). The effects of dietary mannanoligosaccarides on cecal parameters and the concentrations of enteric bacteria in the ceca of Salmonella-challenged broiler chicks. *Poultry Sci*., 79: 205-211.
22. Swenson, D.L.; Clegg, S.; Old, D.C. (1994). Frequency of the fim genes among Salmonella serovars. Microb. *Pathogen*., 10: 487-490.
23. Tinker, J.K.; Clegg, S. (2000). Characterization of FimY as a coactivator of type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. *Infect. Immun*., 68: 3305-3313.
24. Tinker, J.K.; Hancox, L.S.; Clegg, S. (2001). FimW is a negative regulator affecting type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol*., 183: 435-442.
25. Truszczynski, M.; Osek, J. (1987). Occurrence of mannose resistant hemaggutinins in *Escherichia coli* strains isolated from porcine colibacillosis. *Comp. Immun. Microbiol. Infect. Dis*., 10: 117-124.
26. Tzortzis, G.; Goulas, A.K.; Gee, J.M.; Gibson, G.R. (2005). A novel galactooligosaccharide mixture increases the Bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colocnic contents of pigs in vivo. *J. Nutr*., 135: 1726-1731.