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In vitro evaluation of the binding capacity of *Saccharomyces cerevisiae* Sc47 to adhere to the wall of *Salmonella* spp

Luis Salvador Pérez-Sotelo,* Martín Talavera-Rojas,* Humberto G. Monroy-Salazar,* Salvador Lagunas-Bernabé,* José A. Cuarón-Ibargüengoytia,** Roberto Montes de Oca Jiménez,* Juan Carlos Vázquez-Chagoyán*

ABSTRACT. *Saccharomyces cerevisiae* Sc47 (*Biosaf*TM) is a commercially available baker's yeast strain (Lesaffre, France) that has been used as a probiotic in animal nutrition. It has been previously reported that animals fed with the yeast showed an improved resistance to several enteric infectious diseases. Some of the *S. cerevisiae* strains adhere potentially pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp. This could be a mechanism through which animals fed with the yeast may become more resistant to infections caused by these microorganisms. In this paper, the adhesion of forty-five *Salmonella* spp. isolates to Sc47 was assessed by sedimentation and agglutination tests, and by light and electron microscopy. Results showed that 57.7% (26/45) of the isolates and 66.6% (6/9) of the *Salmonella* serovars tested adhered to the Sc47 cell wall.

Key words: *Saccharomyces*, *Salmonella*, Sc47, agglutination, probiotic.

INTRODUCTION

Salmonella spp. is the second most common etiological agent isolated from enteric diseased pigs. In most cases, it is the cause of moderate diarrheic enteritis in piglets, and is one of the main infectious agents in human enteric zoonoses, as it is found in meat and food.^{26,27} A wide range of antibiotics are used to treat human salmonellosis. However, genetic mutations and selective pressure have pushed *Salmonella* spp., as well as other bacteria, to become resistant or multi-resistant to antibiotics.^{2,3} Furthermore, the use of subtherapeutic doses of antibiotics as growth promoters in the pig industry may have accelerated the wide spread of antibiotic resistant strains.¹⁹ Hence, the development of al-

RESUMEN. La cepa Sc47 (*Biosaf*TM) de *Saccharomyces cerevisiae*, es una levadura de panadería bajo patente industrial (Lesaffre, Francia), que ha sido usada como probiótico en la alimentación animal. Se ha observado que los animales que reciben esta levadura en la dieta tienen una mayor resistencia a diversas enfermedades infecciosas. Algunas cepas de *S. cerevisiae* adhieren bacterias potencialmente patógenas como *Escherichia coli* y *Salmonella* spp a su pared. Lo anterior sugiere un posible mecanismo por el cual los animales que consumen la levadura presentan una mayor resistencia a estas infecciones. En el presente estudio, se evaluó la adherencia de 45 aislamientos de *Salmonella* spp. a la cepa de *S. cerevisiae* Sc47 mediante pruebas de aglutinación, sedimentación, y de microscopía óptica y electrónica. Los resultados obtenidos mostraron que el 57.7% (26/45) de los aislamientos de *Salmonella* spp. y el 66.6% (6/9) de sus serovares mostraron adherencia a la levadura en cuestión.

Palabras clave: *Salmonella*, *Saccharomyces*, Sc47, aglutinación, probiótico.

ternative therapeutic or prophylactic agents, such as probiotics, have been under intense study for the last decade. Some *Saccharomyces cerevisiae* strains have been used as probiotics in humans for many years, because they exert some influence on the intestinal flora with efficacy in clinical trials for prevention and treatment of antimicrobial-associated diarrhea.^{17,10} It has been reported that active yeasts, such as *Saccharomyces cerevisiae* *boulardii* (*hansen CBS 5926*) improve the resistance of the intestinal ecosystem to infectious pathogens such as *Clostridium difficile*,^{9,8,28} *Escherichia coli*,¹² *Shigella flexneri*,²⁵ *Vibrio cholerae*,^{4,5} *Candida albicans*¹⁰ and *Salmonella typhimurium*.¹² In addition to its therapeutic and prophylactic use in humans, *S. cerevisiae* has been used for over a decade as a probiotic in pigs because of its effect on growth and reproductive performance and the reduction of morbidity and mortality, particularly in young animals. Several *S. cerevisiae* strains are commercially available and have been used in the animal production industry.^{34,20} Some of the mechanisms that may help to understand how yeasts are able to protect the hosts against pathogens include the stimulation of the immune system at the lymphoid tissue level in the gut,²² degradation of bacterial toxins by the yeast's pro-

* Centro de Investigaciones y Estudios Avanzados en Salud Animal (CIESA), Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México.

** CENID-Fisiología Animal, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Ajuchitlán, Querétaro.

teolytic enzymes,⁶ inhibition of bacterial adherence to gastrointestinal epithelial cells by the release of a protease that digests the bacter receptor for certain pathogens such as *Cl. difficile*,⁶ and formation of yeast-bacter conglomerates by bacterial adhesion to the yeast's cell wall.^{11,21,23}

There are many different strains of *S. cerevisiae* but only a few have been studied for their probiotic properties or for their capacity to adhere bacteria to their wall, and not all *S. cerevisiae* strains have the same beneficial effects on health and productive parameters.^{31,32} Previous studies have compared *S. boulardii* properties and some other probiotic strains, and showed that *S. boulardii* could bind *E. coli* type I fimbriae (FIM) more strongly than other *S. cerevisiae* probiotic strains.^{12,15}

S. cerevisiae Sc47 (Biosaf®, Saf-agri, Toluca, Mexico) is a commercially available strain, originally intended for the food industry. Its fermentation characteristics and cell wall composition prompted its testing as a potential probiotic. It has been used for over 20 years as a probiotic in pigs¹⁴ and other livestock animals^{7,8} for its effect of reducing the frequency of diarrhea and other diseases in treated animals.²⁰ In field trials, some protective effects against pathogenic *E. coli* and *Isospora suis* have been observed in Sc47-fed pigs (Trujano M., personal communication). Even though *S. cerevisiae* Sc47 has been studied for several years, the mechanisms involved in pig disease protection have not been studied. In our laboratory, we are interested in the mechanism by which *S. cerevisiae* Sc47 prevents *Salmonella* adhesion to the intestinal epithelium. It is the aim of this work to test the capacity of Sc47 to adhere *Salmonella* spp to its wall.

MATERIAL AND METHODS

Test bacteria

Forty-seven *Salmonella* spp. isolates (classified as *Salmonella enterica* subspecies *enterica* (Table 1) were used in this study. Forty-five isolates were randomly obtained from a commercial abattoir from healthy looking pigs of unknown salmonella status; samples were collected from gall bladder or mesenteric lymph nodes. Isolates were serotyped at the Instituto Nacional de Diagnóstico y Referencia Epidemiológica (INDRE), Mexico.²⁹ Out of the 45 isolates, 9 different serovars were identified and all 45 isolates were motile. All bacterial strains were individually cultured in brain and heart infusion (BHI) broth (Difco, USA) at 37°C for 24 h. Bacterial cultures were adjusted to a concentration of 8×10^9 CFU (colony-forming units)/ml (Spectronic 21D, Milton Roy at 540 nm) before exposure to Sc47.^{24,33} Additionally, a non-motile *Salmonella gallinarum* chicken isolate isolated in our laboratory and the motile strain ATCC 14028 were used as negative and positive controls, respectively.

Saccharomyces cerevisiae Sc47

Saccharomyces cerevisiae Sc47 is commercially marketed as Biosaf™ (Safmex S.A. de C.V, km 57.5 Carretera México-Toluca, Toluca Estado de Mexico) and was originally isolated from a proprietary germoplasm bank (Societe Industrielle Lesaffre 137 Rue Gabriel Peri-BP 3629, 59730 Marcq-en-Baroeul Cedex, France) of *Saccharomyces cerevisiae*. Biosaf™ has a concentration of 1×10^{10} cfu/g. For *in-vitro* assays, it was rehydrated with PBS (pH 7.2) 1:10 (w/v) at room temperature, and adjusted to a concentration of 8×10^7 CFU/ml.

Salmonella adhesion to Sc47 cell wall assays

Ten randomly chosen field isolates, plus a reference strain (*Salmonella* spp. ATCC 14028) and a *S. gallinarum* isolate, were assessed for adhesion by sedimentation and agglutination tests. From these, four isolates (three *S. typhimurium* and one *S. choleraesuis*), ATCC 14028 and the *S. gallinarum* isolate were included as controls for this and all the other experiments.

Sedimentation assay

Sc47 was rehydrated in 1 ml of PBS (pH 7.2) and adjusted to 8×10^7 CFU/ml. Five hundred µl of this suspension was placed in a 1.5 ml conical microcentrifuge tube and 50 µl of an overnight culture of the testing bacteria containing

Table 1. *Salmonella* spp. vs. Sc47: sedimentation and adhesion assay with or without mannose.

Salmonella serotype*	Proportion of isolates positive for sedimentation and agglutination	Proportion of isolates positive for sedimentation and agglutination in presence of mannose
<i>S. enterica</i> ser. Typhimurium	19/30	0/30
<i>S. enterica</i> ser. Choleraesuis	1/1	0/1
<i>S. enterica</i> ser. Anatum	3/4	0/4
<i>S. enterica</i> ser. Bredeney	1/3	0/3
<i>S. enterica</i> ser. Agona	1/2	0/2
<i>S. enterica</i> ser. Reading	1/2	0/2
<i>S. enterica</i> ser. Monofasica	0/1	0/1
<i>S. enterica</i> ser. London	0/1	0/1
<i>S. enterica</i> ser. Enteritidis	0/1	0/1
Total	26/45 (57.7%)	0/47 (0%)

* *Salmonella* serotypes were identified by slide agglutination test using the Kauffmann-White scheme

** *S. typhimurium* ATCC 14028 and *S. gallinarum* were used as positive and negative controls respectively (data not shown).

approximately 8×10^9 CFU/ml was added to the yeast suspension and mixed by pipetting. The mixture was incubated at room temperature for five minutes and sediments were subjectively evaluated by the pellet size observed at the bottom of the tube. Negative controls included testing bacteria alone, Sc47 alone, a mixture of Sc47 with *S. gallinarum* or BHI broth. The positive control was a suspension containing Sc47 and ATCC 14028 (Fig. 1).

Agglutination assay

The ability of *Saccharomyces* to agglutinate *Salmonella* was evaluated according to a protocol published elsewhere¹⁶ with the following modifications: 10 μ l of a Sc47 suspension containing approximately 8×10^7 CFU/ml were mixed with 10 μ l of an overnight culture of the testing bacteria (8×10^9 CFU/ml) on a microscopic slide using a pipette tip. Agglutination was observed by eye and subjectively evaluated by estimating the extent of binding immediately after the microorganisms were mixed together. Control samples in this experiment were the same as those used for the agglutination test (Fig. 2).

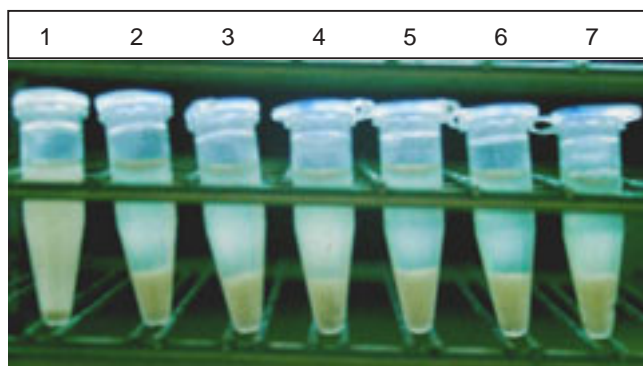


Figure 1. Sedimentation assays in tubes. *Salmonella* spp. sedimentation induced by Sc47 is shown. From left to right, tube 1, negative control: *S. gallinarum* isolate; tube 2, *S. typhimurium* isolate 15; tube 3, *S. typhimurium* isolate 72; tube 4, positive control: *S. typhimurium* ATCC 14028; tube 5, *S. typhimurium* isolate 74 and tube 6, *S. choleraesuis* isolate 21.

Mannose-sensitive adhesion assay

In order to know whether type 1 fimbriae were the main factor involved in the sedimentation-agglutination reactions, all the isolates were tested for mannose sensitive adhesion according to a protocol published elsewhere¹² with the modification that Sc47 was rehydrated in 1 ml of PBS containing 5% (w/v) D-mannose. To the rehydrated yeast (8×10^7 CFU/ml) an equal volume of a pure bacterial culture containing 8×10^9 CFU/ml was added. The final mannose concentration was 2.5%. Controls used were equivalent to those mentioned above. Interpretation of results was also according to the protocols described above.¹²

Adhesion assay

All the agglutination positive slides were Gram stained using a standard method.^{30,32} Smears were observed at 10, 40, and 100X magnifications by light microscopy. Adhesion was subjectively evaluated by observing the amount of bacteria bound to the yeast (Fig. 3, 4).

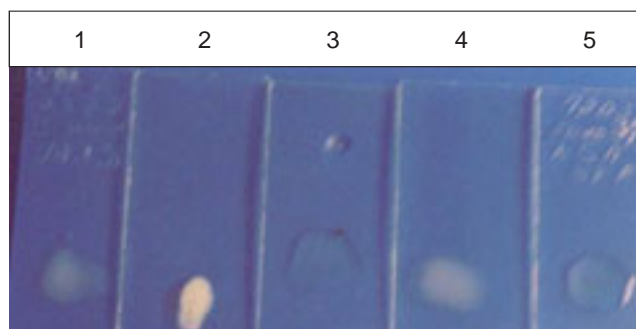


Figure 2. Agglutination test: *Salmonella* spp. agglutination when in contact with Sc47. From left to right slide 1, Sc47 in PBS; slide 2, positive control: Sc47 mixed with *S. typhimurium* ATCC 14028; slide 3, pure culture of *S. typhimurium* in BHI; slide 4, negative control: Sc47 mixed with *S. gallinarum* and slide 5, BHI broth alone.

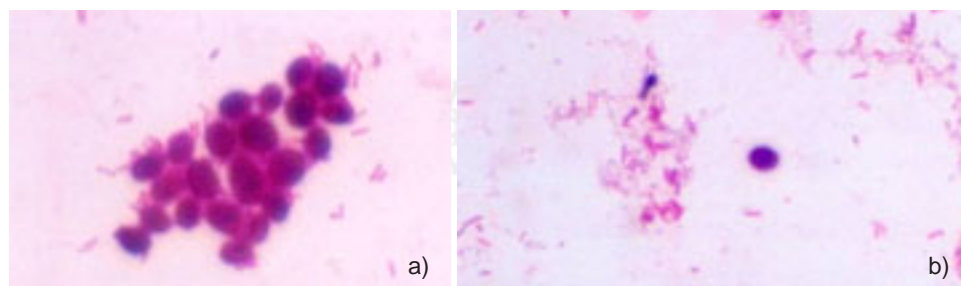


Figure 3. Adhesion test as observed on Gram stained smears. a) positive reaction showing *S. typhimurium* ATCC 14028 adhesion to Sc47 and b) *S. gallinarum* negative adhesion to Sc47.

Transmission electron microscopy

Sample preparation for transmission electron microscopy was done according to a protocol published elsewhere¹³ as follows: 30 µl of yeast suspension (8×10^7 CFU/ml) were washed twice with PBS (pH 7.4), then mixed with a bacterial test culture (60 µl of 8×10^9 CFU/ml) and fixed with glutaraldehyde. Cell membranes were sensitized with osmium tetroxide (OsO_4). Samples were dehydrated with increasing concentrations of a 1:1 solution of ethanol and propylene oxide with epon in a desiccator. After incubation, the samples were embedded in epon (100%). Polymerization was induced by incubation at 60°C for 48 h. Ultrathin sections (700 nm) of the pellets were cut using an ultramicrotome (Reichert-Jung, Austria) and stained with uranyl acetate and lead citrate. Samples were observed with a Jeol 1200 CXII electron microscope.

RESULTS AND DISCUSSION

Six *Salmonella* spp. were used for protocol standardization; four motile *S. typhimurium* isolates (15, 72, 74, 19) from pigs, one motile reference strain (*S. typhimurium* ATCC 14028), and one non-motile *S. gallinarum* isolate from a naturally infected chicken. All the strains and isolates showing positive sedimentation reaction were also positive for agglutination, and bound to the yeast's cell wall as observed by light and transmission electron microscopy, while the *S. gallinarum* isolate (used as negative control) did not induce sedimentation, agglutination or adhesion to Sc47. Interestingly, none of these bacteria showed sedimentation or agglutinating properties in the presence of D-mannose (Table 2).

Of the 45 *Salmonella* pig isolates tested, 57.7% (26/45) induced sedimentation and agglutination when in contact with Sc47, and none (0/47) in the presence of D-mannose (Table 1). It can also be observed in Table 1 that 66.6% (6/9) of the different *Salmonella* serovars used in the present study were able to induce sedimentation and agglutination, although isolates of the same serovar did not necessarily show the same sedimentation-agglutination pattern. Previous reports have shown that *Saccharomyces* has some affinity to *Salmonella* strains that express type 1 fimbriae.¹ This affinity is mediated by the mannan oligosaccharides (MOS) of *Saccharomyces* cell wall, and can be inhibited by mannose complexes.^{12,16} In the present study, more than 50.0% of the *Salmonella* isolates tested bound to *S. cerevisiae* Sc47 and all of them were inhibited by mannose.

It was interesting to observe that isolates of the same *Salmonella* serovar had different abilities to adhere to *S. cerevisiae* Sc47, and that the number of serovars with binding and non binding phenotypes were evenly distributed (approximately 50% of each) (Table 1). Differences in binding capacity have been previously reported by Mc Dermid *et al*²² who stated that growing conditions such as temperature, humidity and pH may have a large impact on *Salmonella* on the capacity of *Salmonella* to express fimbriae. In that study, it was reported that up to 52.0% of the tested strains would express fimbriae if grown at a near-neutral pH (7.1), but they would not express it if grown at extreme pH conditions (4.35 or 9.45). Additionally, another study reported that some biphasic *S. typhimurium* strains (particularly strain 798), were able to frequently switch between FIM⁺ and FIM⁻ phenotypes at a rate of 10^{-2} to 10^{-5} per generation. Experiments in pigs showed that *Salmonella* FIM⁺ strains were able to survive and colonize the pig's intestinal epithelium more efficiently than *Salmonella* FIM⁻

Table 2. Sedimentation, agglutination and adhesion assay protocol standardization.

*Strain/serotype	Sediment.	Agglut.	Gram stained smears (Adhesion)	Transmission electron microscopy (Adhesion)	Sediment** In presence of D-mannose	Agglut**
<i>S. enterica</i> ser. Typhimurium 15	+	+	+	+	-	-
<i>Salmonella enterica</i> ser. Typhimurium 72	+	+	+	+	-	-
<i>Salmonella enterica</i> ser. Typhimurium 74	+	+	+	+	-	-
<i>Salmonella enterica</i> ser. Choleraesuis 19	+	+	+	+	-	-
<i>Salmonella enterica</i> ser. Typhimurium ATCC 14028	+	+	+	+	-	-
<i>Salmonella enterica</i> ser. Gallinarum	-	-	-	-	-	-
PBS (control)	-	-	-	-	-	-

* The serotypes were identified by slide agglutination test using the Kauffmann-White scheme

** Sediment = sedimentation; Agglut. = agglutination

strains. From these results, it has been hypothesized that the adhesion differences between serovars could be explained by *Salmonella*'s capacity to regulate genes involved in FIM expression.¹

In the present study the capacity of Sc47 to bind *Salmonella* spp *in-vivo* was not evaluated, nor were the likely differences in the binding capacity of other commercially available *Saccharomyces* strains tested, so further studies should be conducted in order to address these issues.

Most *Salmonella* serovars in pigs produce a self limiting infection associated with diarrhea,²⁸ while *S. choleraesuis* is able to produce septicaemia.²⁴ In either case, the infection has an oral route and the bacteria have to travel through the intestinal tract to produce the disease. The observation that Sc47 was able to reduce diarrhea frequency in piglets might be related to its capacity to interfere with *Salmonella*, and probably with other bacteria such as pathogenic *E. coli*,

reaching the intestinal epithelium, thus preventing disease. Sc47 may block the *Salmonella* FIM mediated adhesion to the intestinal wall, and consequently diminishing its survival and colonization opportunities. Further studies need to be conducted in order to prove this hypothesis.

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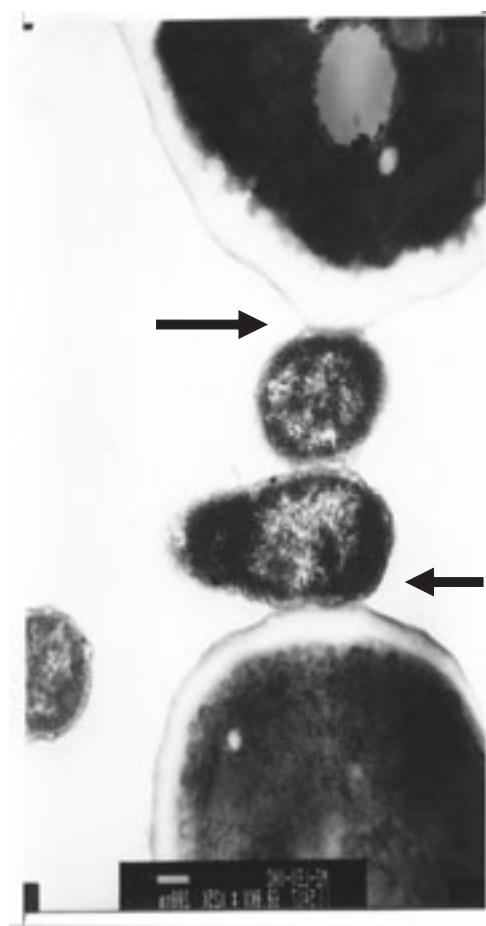


Figure 4. Adhesion test by transmission electron microscopy: arrows show the binding of testing bacteria (*S. typhimurium* ATCC 14028) to Sc47, as observed on a preparation for EM.

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Correspondence to:

M. en SP. Luis S. P  rez Sotelo.
CIESA-FMVZ-UAEM,
Km. 15 de la autopista Toluca-Atlaconulco,
Tel/Fax : 01722-2965555,
e-mail: luismsp09@hotmail.com.mx.