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Production of probiotic lactobacilli biomass for in-feed supplementation to growing pigs and sows

Producción de biomasa de lactobacilos probióticos para suplementación en la alimentación de cerdos en crecimiento y cerdas

Produção de biomassa de lactobacilos probióticos para suplementação na alimentação de suínos em crescimento e matrizes

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Abstract

In the design of probiotic formulations, preservation of microorganism's viability is the most important parameter to evaluate at the time of administration. In this study, the impact of spray-drying on the preservation of probiotic strains isolated from pig feces: *Limosilactobacillus reuteri* CRL2222, *Lactobacillus amylovorus* CRL2225 and *Lactobacillus johnsonii* CRL2229 was studied, by using a previously formulated protectant mix. The three probiotic strains displayed high resistance to spray-drying operating conditions and subsequent storage, showing survivability's between 92.9 and 94.7%; probiotic powders exhibited low water activity (a_w:0.2) and moisture content ranging from 3.3±0.16 and 3.8±0.46. The used trehalose/maltodextrin/soy protein/sodium glutamate/calcium phosphate protectant mix showed high thermal protection during the process, allowing high final viability. When probiotic functionality of the strains was evaluated, hydrophobic character was maintained. Thus, it may be highlight that both viability and functionality of probiotics was preserved after spray-drying, characteristics that are strain-specific.

Key words: Probiotic lactic bacteria, Technological properties, Spray dryer, Probiotic formulations, Pigs

Resumen

En el diseño de formulaciones probióticas, la preservación de la viabilidad de los microorganismos es el parámetro más importante a evaluar en el momento de su administración. En este estudio, se estudió el impacto del secado por aspersión en la preservación de cepas probióticas aisladas de heces de cerdo: *Limosilactobacillus reuteri* CRL2222, *Lactobacillus amylovorus* CRL2225 y *Lactobacillus johnsonii* CRL2229, utilizando una mezcla protectora previamente formulada. Las tres cepas probióticas mostraron una alta resistencia a las condiciones de operación del secado por aspersión y al almacenamiento posterior, con una supervivencia de entre el 92.9 % y el 94.7 %; los polvos probióticos presentaron una baja actividad de agua (a_w:0.2) y un contenido de humedad que osciló entre 3.3±0.16 y 3.8±0.46. La mezcla protectora de trehalosa/maltodextrina/proteína de soja/glutamato de sodio/fosfato de calcio utilizada mostró una alta protección térmica durante el proceso, lo que permitió una alta viabilidad final. Al evaluar la funcionalidad probiótica de las cepas, se observó que se mantuvo su carácter hidrofóbico. Por lo tanto, cabe destacar que tanto la viabilidad como la funcionalidad de los probióticos se conservaron tras el secado por aspersión, características específicas de cada cepa.

Palabras claves: Bacteria láctica probiótica, Propiedades tecnológicas, Secado por spray, Formulaciones probióticas, Cerdos

Resumo

No desenvolvimento de formulações probióticas, a preservação da viabilidade do microrganismo é o parâmetro mais importante a ser avaliado no momento da administração. Neste estudo, estudou-se o impacto da secagem por atomização na preservação de cepas probióticas isoladas de fezes suínas: *Limosilactobacillus reuteri* CRL2222, *Lactobacillus amylovorus* CRL2225 e *Lactobacillus johnsonii* CRL2229, utilizando uma mistura protetora previamente formulada. As três cepas probióticas apresentaram alta resistência às condições operacionais de secagem por atomização e armazenamento subsequente, apresentando sobrevivência entre 92.9 e 94.7%; os pós probióticos apresentaram baixa atividade de água (a_w:0.2) e teor de umidade variando de 3.3±0.16 a 3.8±0.46. A mistura protetora de trealose/maltodextrina/proteína de soja/glutamato de sódio/fosfato de cálcio utilizada apresentou alta proteção térmica durante o processo, permitindo alta viabilidade final. Quando a funcionalidade probiótica das cepas foi avaliada, o caráter hidrofóbico foi mantido. Assim, pode-se destacar que tanto a viabilidade quanto a

funcionalidade dos probióticos foram preservadas após a secagem por atomização, características que são específicas da cepa.

Palavras-chave: Bactérias lácticas probióticas, Propriedades tecnológicas, Secagem por atomização, Formulações probióticas, Suínos

Introducción

Probiotics are viable microorganisms or microbial mixtures able to colonize the intestine that are administrated to improve human and animal microbiota balance. Probiotics administration contributes to the establishment of a beneficial intestinal population in the host serving as antagonist for disease-causing bacteria and able to modulate gut microbiota and immunological systems in both humans and livestock.[1,2] Recently, the administration of probiotics to meat animals from intensive farming systems has been widely evaluated because of their ability to prevent diseases, accelerate animal growth, and optimize reproduction rates.[2] Particularly for swine rearing, piglets are severely affected by the stress occurring after weaning that leads to significant economic losses for pig farmers.[3] To counteract these negative effects, antibiotics feed additives have long been used as therapeutic alternatives and growth promoters. However, the development/spread of antimicrobial-resistant bacteria, which may threaten the health of animals and consumers of animal products, led to their ban in meat animal production. [4] Therefore, probiotics have been reported as desirable alternative to antibiotic growth promoters, supporting swine health (post-weaning diarrhea) and growth increase. [5] Many studies reported on the administration of probiotics involving yeasts and bacteria species. Among the bacterial probiotics administered to farm animals, lactic acid bacteria (LAB) as lactobacilli, enterococci, and pediococci were the most used. [6,7]

According to FAO/WHO, products claiming probiotic effects should contain enough number of viable cells to confer efficacy and benefits, while ensuring their properties throughout shelf-life as prerequisite for their administration, most of them related to specific strains, even though they are taxonomic classified into the same bacterial specie or genus. However, given the huge/large scale of animal production systems, it is required to deliver high numbers of probiotic live bacteria; biomass must be produced in optimized low-cost media and cultivation conditions using appropriate fermentation procedures. It is known that LAB are nutritionally exigent organisms; the presence of key ingredients must be assured in the culture media. [8,9,10] The industrial application of LAB probiotics depends on biomass concentration and preservation technologies to guarantee long-term stability in terms of viability and functional activity. For long-term storage

water must be eliminated, cell harvesting and concentration after fermentation is commonly carried out by centrifugation or membrane filtration. Even when freeze-drying is the most conventional process used for industrial production of dried bacteria, in terms of cost and production scale, successful applications of spray-drying for producing probiotic cultures have been reported. [11,12] The difference between these processes is the fast and hot air current used to dehydrate small, atomized droplets of bacterial cell suspensions in spray-drying. Dehydration and temperature are the main responsible for viability deterioration during drying of bacteria; the extent of bacterial inactivation depends on the temperature-time combination.[13] However, the use of effective protectant/carrier formulations during spray-drying of LAB is one of the most important factors affecting their survival and is highly dependent on the strain and type of protectant [14] Because drying processes strongly affect not only cell viability but also probiotics function[15], the influence of spray-drying on cell functionality is not directly related to bacterial cell survival but is strain dependent. In view to probiotic administration to neonatal and weaning piglets as well as pregnant sows, suspensions and capsules containing spray-dried probiotic/s are often used. Thus, in this study, the effect of spray-drying on the viability and maintenance of functionality of previously selected probiotics *Limosilactobacillus* (*Lim*.) *reuteri* CRL2222, *Lactobacillus* (*L*.) *amylovorus* CRL2225 and *Lactobacillus* (L.) *johnsonii* CRL2229 strains was evaluated.[16]

Material and Methods

Microorganisms and culture conditions

Limosilactobacillus (*Lim.*) *reuteri* CRL2222, *Lactobacillus* (*L*.) *amylovorus* CRL2225 and *Lactobacillus* (*L*.) *johnsonii* CRL2229 strains previously isolated from feedlot cattle and characterized for their probiotic potential. [16] The inoculum was prepared by transferring glycerol stock culture to MRS broth (Biokar Diagnostics, Beauvais, France) and sub-cultured twice in the same media at 37 ºC for 18 h.

Probiotics biomass

After sub-culturing, each probiotic strain was inoculated (2%) in 1 L MRS broth (Biokar, France) in a Schott-type flasks and incubated at 37 ºC for 18 h. Biomass from each probiotic strain culture was obtained by centrifugation (8.000 g, 10 min at 4 °C) (Sorval RC-6 Plus, Thermo Scientific, Germany), washed twice with saline solution (SS; 0.85%) and centrifuged again.

Drying medium formulation

Cell pellets of each probiotic strain were processed individually. To obtain dry powder for each probiotic lactobacilli, each pellet was resuspended in 500 ml of drying medium/matrix used as a protector (w/w): 15% trehalose (Treh, Anedra, Argentina), 5% maltodextrin DE-10 (Cibart, Argentina), 1% soy protein isolates (Indias, Argentina), 1.25% monosodium glutamate (Centauro Alpha, Argentina) and 3% tricalcium phosphate (Granotec, Argentina).

Probiotic lactobacilli spray-drying

Drying of probiotic strains was performed in a mini spray-drying (Büchi, B-290, Switzerland) and parameters were adjusted to get the least viability loss, the lowest water activity (a_w) and residual moisture to warranty stability during storage. Spray-drying conditions were Ti (inlet temperature) 135 °C, To (outlet temperature) 70-75 °C, feed flow rate 10-12 ml/min, drying air flow rate 35 m^3/h, nozzle (cleaning needle) 6, and spray air flow 610 L/h. The drying conditions used were defined based on the working group's previous experience with lactic acid bacteria, particularly with strains of the same genus and closely related to those used in this study.[17] Water activity (a_w) of dried probiotic biomass was determined using an AquaLab 4TE hygrometer (Decagon Devices Inc., USA), which operates based on the chilled mirror dew point method. This method directly measures aw by determining the condensation temperature of water vapor in equilibrium with the sample and is considered a primary, highly accurate, and reproducible technique [18,19] Regarding calibration, manufacturer-recommended standards were used, including activated carbon (aw < 0.5) and distilled water (aw ≈ 1.0), which allow verification of instrument performance at the extremes of the measurement range. Although the aw values of the analyzed samples were within an intermediate range, the use of these standards ensures instrument linearity and accuracy across the full operational range. [18] However, current practices recommend the use of calibration standards that bracket the specific aw range of the samples in order to improve measurement accuracy within the region of interest. [20,21] In this regard, although intermediate standards were not employed, the applied methodology is validated and widely accepted for food and biological matrices, ensuring reliable results. [22] Subsequently each sample (1 g) was introduced into the chamber and the reading recorded. Residual moisture of bacterial biomass after spray-drying was assessed using a moisture analyser (OHAUS MB 35 Scale, Argentina) at a temperature of 105 °C. The dried bacterial biomass of each probiotic strain was aseptically packed in trilaminate aluminium bags and sealed under vacuum (80%) (Turbovac 120, Argentina). The bags were stored at 4 °C and viability were determined before administration to animals.

The strains powders were later included in N°1 gelatine capsules for adult animals. To the newborn piglets, 3% alginate was added to small bottles, resuspended with sterile water, and orally administered to piglets in 1 ml syringes. Procedures and protocols used in this study were

approved by the Institutional Committee for the Care and use (Res: ID CICUAE 05-21/04-22. Date: April 2022).

Resistance of probiotic lactobacilli to spray-drying process

Evaluation of probiotic resistance to spray-drying was assessed by counting the number of viable cells before and after the process. Successive dilutions were prepared in sterile SS and subsequent plating on MRS agar. Plates were incubated for 48 h at 37 ºC and colonies number determined. Survival rate (%) was expressed as N/Ni, in which N: log CFU/mL after treatment and Ni: log CFU/mL before treatment. Survivability rates as percentage was also calculated.

Maintenance of probiotic properties after spray-drying

To determine the effect of spray drying on probiotic functionality, surface properties were also evaluated 6 months post-drying. Surface hydrophobicity was assessed and quantified by determining the change in optical density at 600 nm (OD_{600nm}) of cell suspensions in physiological saline solution after partitioning with organic solvents [23] by adding xylene and toluene, as modified by Ocaña et al. (1999) [24]. The pellet was washed twice with saline solution (0.85% NaCl), centrifuged, and resuspended at an initial OD_{600nm}=0.60±0.06 (OD0) (Spectronic 20, Bausch and Lomb, Rochester, New York, USA). To tubes containing 3.6 ml of bacterial suspension, 0.6 ml of hydrophobic solvent (xylene and toluene) (Cicarrelli, Argentina) was added (solvent-bacterial suspension ratio: 1/6). The tubes were shaken for 1 minute, and the final OD_{600nm} (ODf) was determined again after 15 minutes of shaking.

Hydrophobicity (%) was calculated using the following formula [25]: Eq. (A) % Hydrophobicity: [(OD0 - ODf) / OD0] x 100 (1)

Self-aggregation was determined using the technique described by Vandevoorde et al. (1992) and modified by Ocaña and Nader Macías (2003) [26]. The strains under study were centrifuged and washed three times in saline solution, and the OD_{600nm} was adjusted to 0.60±0.05 (OD0). The suspensions were allowed to settle to determine the OD_{600nm} at different time periods (1, 2, 3, 4, and 24 h) (ODf). The percentage of self-aggregation was calculated using an expression similar to that used to determine the percentage of hydrophobicity. The determination of autoaggregation and hydrophobicity was performed using two independent assays, with each sample analyzed in triplicate.

Statistical analysis

The mean values and the standard deviation were calculated from the data obtained with triplicate trials. The results were subjected to an analysis of variance (ANOVA) using a general linear model (GLM) to determine if the mean Log CFU counts of each strain tested differed pre-

and post-spray drying, and to assess survival (%) and stability among the different strains. A significance level of 5% ($p \leq 0.05$) was applied, followed by a Tukey test. Minitab (version 21.4.1) was used as the analytical software.

Results and discussion

Because probiotic supplementation to animals demands high viability, the maintenance of adequate levels of viable cells and functionality after dehydration by spray-drying throughout shelf-life is a prerequisite for their administration to animals. MRS culture medium for biomass obtainment, and the mix trehalose/maltodextrin/soy protein isolate/monosodium glutamate/$(PO_4)_2Ca_3$ as carrier/protectant was used, while cell mass dehydration was performed by a laboratory spray-dryer. Under the used operating conditions, cell suspensions containing viable cells between 9.6±0.12 and 9.8±0.08 log total CFU before spray-drying (after growth in MRS), achieved between 9.1±0.56 and 9.2±0.50 log total CFU immediately after spray-drying process, indicating a survivability between 92.8±7.01 and 94.7±7.11% (Table 1).

Table 1. Probiotic lactobacilli cell viability and stability after spray-drying

Probiotic strain	Cell viability (log total CFU)		urvival (%)	a_w	Residual moisture (%)	Stability• log CFU/g)
	Before drying	After drying				
***Lim. reuteri* CRL2222**	9.8±0.08^{a}	9.1±0.56a*	92.8±7.01^{C}	0.2±0.02	3.8±0.46	7.5±0.00
***L. amylovorus* CRL2225**	9.6±0.12^{a}	9.1±0.64^{a}	94.7±5.33^{A}	0.2±0.04	3.6±0.17	9.0±0.05
***johnsonii* CRL2229**	9.8±0.15^{a}	9.2±0.50a*	93.8±3.21^{B}	0.2±0.05	3.3±0.16	8.2±0.1

a_w: water activity, • Stability determined after 60 days of storage at -20 ºC.

Data are expressed as the mean Log total CFU ± standard error of the biomass before and after spray drying. Rows with different lowercase letters indicate significant differences ($p < 0.05$) in the Log total CFU of the microbial biomass before and after spray drying. Columns with different uppercase letters indicate significant differences ($p < 0.05$) in post-spray drying survival between strains, according to Tukey's test. Asterisks (*) indicate significant differences ($p < 0.05$) in the stability of the strains 60 days after spray drying, according to Tukey's test.

LAB are known to adapt to the progressive reduction in pH through sophisticated mechanisms at physiological and molecular level. [27] Indeed, *Lactobacillus* species have been considered intrinsically resistant to acid, increasing their survival in the presence of metabolizable sugars. In addition, exposure of probiotic lactobacilli to sub-lethal acid stress before the drying process can increase their stability during storage. [28] After spray-drying, probiotic powders exhibited low water activity (a_w: 0.2) and moisture content ranging between 3.3±0.16 and 3.8±0.46, while stability after 60 days of storage at **4 ºC** tended to decrease in a strain-dependent manner. A viability loss (CFU/g) between 0.7 and 0.5 log units after storage was observed, being greater for *Lim. reuteri* CRL2222 probiotic strain. These results agree with spray dried *L. acidophilus* NCIMB701748 which exhibited minimal viability loss [29]; indicating is a required characteristic of the strains for their long-term storage and good handling. [14]

The operating conditions used and appropriated carriers/protectants allowed the production of dried probiotic lactobacilli with high viability (>9 log CFU/g) and a survivability between 92.9 and 94.7%, indicating the resistance of the three probiotic strains to drying process conditions. Similar viability values after spray-drying were reported for probiotics *Lacticaseibacillus paracasei* 431 and *Lim. fermentum* CRL1446/*L. johnsonii* CRL1231 using whole milk matrix and sodium alginate/inulin/maltodextrin mix as protectant, respectively. [30,17] Similarly, the presence of lactose and trehalose showed low reductions after spray-drying of *Lactobacillus rhamnosus* GG. [31] The evaluated effect of protectants such as arabic gum, maltodextrin, gelatine on the survival of *Lim. reuteri* LR92 in fermented vegetable pulp after spray-drying showed higher survival in the presence of gelatine. [32] On the contrary, recent results showed significant reduction in viability from 10^9 to 10^7 CFU/g when strains of *L. acidophilus* and *Lpb. plantarum* isolated from piglet feces were spray-dried using maltodextrin/glucose as protective matrix.[33] The use of effective protectant/carrier formulations during spray-drying of LAB is one of the most important factors affecting their survival and is highly dependent on the strain and type of protectant.[14] In this study, probiotics lactobacilli strains greatly resisted spray-drying conditions and high cell viability was reached with the use of trehalose/maltodextrine/soy protein isolate/monosodium glutamate/$Ca_3(PO_4)_2$ as protectant mix. Savedboworn et al. (2019) [34] reported the synergistic effect of combining protein and sugars on cell survival, rather than acting individually. The disaccharide trehalose used at a 15% concentration in the carrier mix was described as highly protective for probiotic lactobacilli cell membranes by replacing water and stabilize in the dry state[35], while maltodextrin increases cell suspension volume by adding solids and functioning as a thickener. [36] In addition, $Ca_3(PO_4)_2$ reduces cell wall damage during drying process by the increase of surface proteins content and is a cell anti-caking agent. [37,5]

The survival of bacteria during spray-drying and powders storage are influenced by many factors including bacterial strain, carrier material, drying temperature and heat exposure time as well as storage conditions. [38] Intrinsic tolerance of bacterial strains is highly strain-dependent, playing a critical role in overcoming their inactivation due to drying injury and storage-related stresses. [11] As reported by Wang and Mutukumira (2022) [39] cell death during spray-drying is mostly caused by changes in the configuration and profile of lipids in the cell wall and cytoplasmic membrane. High temperatures affect essential cellular components, among which ribosomes have been reported to be critical components.[38] Dehydration and temperature are the two factors responsible for viability deterioration during drying of bacterial cultures; however, the extent of bacterial inactivation depends on the temperature-time combination[13] Therefore, the optimum residence time must be that for complete removal of moisture with minimum increase in the temperature of the dried products.[38] In this study, 135 ºC and 70-75 ºC as inlet and outlet air temperatures were applied for spray-drying of probiotic lactobacilli, which exhibited similar viability before and after the process in agreement to that reported for *Lim. reuteri* DPC16 that indicated the highest cell counts (98%) with 0.19 a_w using 160°C/80°C as inlet/outlet temperatures. [39] Similarly, *L. johnsonii* CRL1231 showed high survival after spray-drying using 130 ºC/80-85 ºC as inlet/outlet air temperatures. [17]

It is known that drying processes strongly affect not only cell viability but also the functionality of probiotic strain[15]; the influence of spray-drying on cell functions being not directly related to bacterial cell survival but is strain dependent. In this study, the effect of spray-drying on probiotic lactobacilli surface properties was evaluated six months after drying and compared with those previously determined. [16] Table 2 shows a comparative reduction in self-aggregation values for *Lactobacillus amylovorus* CRL2225 and *Lactobacillus johnsonii* CRL2229, while probiotic *Limosilactobacillus reuteri* CRL2222 exhibited an increase in its self-aggregative ability after drying. Regarding cell surface properties, hydrophobicity values were higher in xylene for all three analyzed strains compared to their initial measurements, whereas a reduction was observed for CRL2225 and CRL2229 in toluene. Overall, an increased hydrophobic character was detected in most probiotic lactobacilli, with the exception of *Lim. reuteri* CRL2222, which displayed alterations in both surface properties, suggesting a decrease in hydrophobicity after drying.

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Table 2. Superficial properties of probiotic lactobacilli after spray-drying

Probiotic strains	Superficial properties (%)					
	Self-aggregation*		Hydrophobicity			
			Xilene		Toluene	
	After drying	Origin values	After drying	Origin values	After drying	Origin values
***Lim. reuteri* CRL2222**	10.0±0.1^{A}	8.3±0.0^{B}	16.9±0.0^{A}	2.0±0.1^{B}	22.2±0.0^{A}	13.0±0.0^{B}
***L. amylovorus* CRL2225**	53.1±0.1^{B}	74.2±0.2^{A}	42.0±0.1^{A}	16.0±0.0^{B}	9.99±0.0^{B}	13.0±0.0^{A}
***L. johnsonii* CRL2229**	46.5±0.1^{B}	67.9±0.0^{A}	40.0±0.1^{A}	10.0±0.1^{B}	34.0±0.0^{B}	62±0.1^{A}

* Self-aggregation at 2 h. •Original values prior to spray drying (Uezen et al., 2023).

Data are expressed as mean percentage (%) ± standard error values for self-aggregation and hydrophobicity. Different capital letters indicate significant differences ($p < 0.05$) in the percentage of self-aggregation and hydrophobicity of the different strains, according to Tukey's test.

The adhesion capability of bacterial cell surface structures can be modified during drying processes, potentially affecting cellular functionality without necessarily compromising probiotic viability. In this regard, Vinderola et al. (2011) [12] reported that technological processes may alter functional surface traits while maintaining cell survival. Similarly, Kiekens S et al. (2019) [39] demonstrated that spray-drying induced modifications in cell wall components of *Lactobacillus rhamnosus* GG, including changes in lipoteichoic acids and disruption of exopolysaccharide biosynthesis, which did not affect viability but significantly impaired adhesion to intestinal epithelial cells. These findings support the hypothesis that alterations in cell wall properties, including surface charge and macromolecular composition, may contribute to increased resistance to drying processes while preserving high viable counts. In relation to these observations, the changes detected in autoaggregation and hydrophobicity for *Lim. reuteri* CRL2222 could have implications for its colonization capacity in the host. Adhesion to the intestinal epithelium is a key criterion in probiotic selection, as it promotes transient persistence, host interaction, and competitive exclusion of pathogens. Autoaggregation has been linked to

microcolony formation and maintenance of high bacterial densities on the intestinal mucosa, while hydrophobicity plays a role in the initial interactions between bacterial cells and mucosal surfaces. Therefore, the modifications observed after drying could translate into altered adhesion efficiency and, consequently, a potential impact on probiotic performance. Nevertheless, the relationship between hydrophobicity, autoaggregation, and adhesion is not strictly linear, as additional factors such as surface proteins, exopolysaccharides, and structures like the S-layer also play critical roles. Furthermore, although the determination of surface characteristics based on hydrocarbon adhesion remains controversial, hydrophobicity measurements are still widely used as an indicator of bacterial surface properties following drying processes.[15] Thus, while the observed changes suggest that drying may influence functional traits associated with colonization, these results should be interpreted with caution. Additional studies, including in vitro adhesion assays using intestinal epithelial cells and in vivo evaluations, are necessary to determine the actual biological relevance of these modifications.

Conclusions

The results of this study showed spray-drying as a suitable method to obtain probiotic bacteria in powder form for administration to piglets and sows. Probiotic lactobacilli were resistant to the drying process and stable for **60 days**, showing concentrations greater than 6 log CFU/g, value required for their use as probiotics, and their hydrophobic character was maintained.

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The data are included in the CONICET repository.

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