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Growth of *Lactobacillus rhamnosus* 64 in whey permeate and study of the effect of mild stresses on survival to spray drying

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ABSTRACT

In this study, we aimed at optimizing the growth of *Lactobacillus rhamnosus* 64 in whey permeate supplemented with different ingredients and we also studied the effects of mild stresses on the response to heat and oxidative challenge. Supplementation of diluted whey permeate with yeast extract, tryptone, Tween 80 and Mn–Mg produced ca. 10^{10} CFU/mL of *L. rhamnosus* 64 under pH controlled fermentation in anaerobiosis. Cells were exposed to different mild stresses (aerobic: 1 h air 0.3 vol vol^{-1} min, pH 6, 37 °C); heat (1 h, pH 6, 45 °C, anaerobiosis) and acid (1 h, pH 5, 37 °C, anaerobiosis), followed by heat (55 °C) or oxidative (0.4 mmol/L H_2O_2) challenge (15 min). Oxidative challenge was more detrimental compared to heat challenge. Anaerobic incubation, cell harvesting at the stationary phase and mild heat stress were more effective for reducing cell death towards heat challenge, whereas cheese whey-starch was effective towards cell protection to spray drying and storage. No effects on cell survival to spray drying were observed when mild stresses were used to increase resistance to spray drying.

1. Introduction

Lactic acid bacteria (LAB) are largely used in the food industry for the production of fermented foods, in particular fermented dairy products where LAB are used to conduct lactic acid fermentation (starter cultures) or due to their capacity to induce, in a strain-specific manner, a health benefit on the host (probiotic cultures) (Carr, Chill, & Maida, 2002). Health benefits induced by probiotics are dose and strain-dependent but in the majority of cases health benefits are observed in commercial products containing 10^7–10^8 CFU/mL (Gueimonde et al., 2004; Raeisi, Ouoba, Farahmand, Sutherland, & Ghoddusi, 2013). This range agrees with the international trend or scientific consensus suggesting that probiotic food should contain 10^7–10^8 CFU/mL to exert a health benefit (Champagne, Gardner, Roy, 2005). Supporting this criterion, positive effects on health were observed in human clinical trials when products containing these concentrations of probiotics were used (Montrose & Floch, 2005). To achieve these amounts of probiotics in a product, concentrated cultures containing 10^{10}–10^{11} CFU/g are required in the dairy industry. Thus, in order to produce economically feasible cultures for industrial applications, the culture medium for biomass production must be optimized for high biomass yield and reduction of production costs of the specific strain. Additionally, cultures must be produced in a suitable format for storage and transport. Probiotic cultures used in functional foods are generally delivered in dried or deep-frozen forms. During the manufacture of dairy products, a variety of by-products such as cheese and ricotta whey or whey permeate are produced, which possess a relatively high organic load due to their content of lactose and proteins (Prazeres, Carvalho, & Rivas, 2012). These by-products, properly managed and supplemented, can be used as growth substrate for the production of probiotic bacteria (Aguirre-Ezkauriatza, Aguilar-Yáñez, Ramírez-Medrano, & Alvarez, 2010; Burns, Vinderola, Molinari, & Reinheimer, 2008). In order to produce dried biomass of functional cultures, freeze-drying is by far

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the most applied technology at the industrial level. Other drying technologies include spray drying, vacuum drying or fluidized bed drying (Muller, Ross, Fitzgerald, & Stanton, 2009). In particular, spray drying is a continuous process and also an attractive low-cost alternative to freeze drying, indeed 6 times cheaper -every Kg of water removed- compared to freeze drying (Knorr, 1998). However, its success (in terms of high cellular viability) is highly strain-dependent (Ananta, Volkert, & Knorr, 2005; Gardiner et al. 2000) as cell dehydration by spray drying implies harsher conditions than freeze drying, where heat and oxidative challenges are the main barriers to cell viability. A fact that makes spray drying an attractive technology for the production of probiotic cultures is the high availability of industrial spray driers at many different scales, since spray drying is an operation heavily used for the production of dried skim milk and food ingredients. For instance, in Argentina, many medium to big-size dairy industries possess spray dryers, which could be exploited for the production of dried probiotics. In a previous work (Lavari, Páez, Cuatrin, Reinheimer, & Vinderola, 2014), cheese whey supported the growth of *Lactobacillus rhamnosus* 64 and acted as a carrier, combined with starch, for the production of dried cultures by spray drying. Nevertheless, a higher concentration of biomass in dairy by-products culture media and enhanced survival along storage are still desirable for this strain for future applications as a probiotic. Considering that sub-lethal stress during production can be useful to improve resistance of probiotics (Foligné, Daniel, & Pot, 2013), the aim of this study was to optimize the growth of *L. rhamnosus* 64 in supplemented whey permeate and to study the effects of mild stresses on the response to heat and oxidative challenge and to survival to spray drying.

2. Materials and methods

2.1. Bacterial strain

L. rhamnosus 64 was isolated in our laboratory from feces of neonates (Vinderola et al. 2008), showed the capacity to promote intestinal IgA in mice (Gregoret, Perezlindo, Vinderola, Reinheimer, & Binetti, 2013), to grow in dairy media and its survival to spray drying in cheese whey-starch as a carrier (Lavari et al. 2014). Overnight cultures (16 h, 37 °C) were obtained in MRS (de Man, Rogosa and Sharpe) broth (Biokar, Beauvais, France) or in a complex basal medium (WMB, Zotta et al. 2012), as specified below, after three successive transfers from frozen (−70 °C) stocks maintained in MRS-18% (w/v) glycerol (Ciccarelli, Santa Fe, Argentina).

2.2. Whey permeate

Partially defatted diluted whey was obtained from a dairy plant producing pasta filata cheese and treated by tangential ultrafiltration (cut-off 20,00 kDa) using a plate and frame unit (Sartocon Midi, with Sartocon cellulose triacetate membranes, model 3021454907E-SG). The permeate (26.8 g/L of lactose monohydrate and 2.36 g/L of galactose, measured using an enzymatic kit, see below) was stored at −20 °C until used.

2.3. Growth of L. rhamnosus 64 in whey permeate

A 2^{5-1} fractional factorial experiment was used to evaluate the effect of yeast extract (0–2.5 g/L), tryptone (0–5 g/L), $NaHCO_3$ (0–1.25 g/L), Tween 80 (0–0.5 mL/L) and Mn–Mg solution (0–10 g/L) on the growth of *L. rhamnosus* 64 to formulate an improved whey permeate medium (Supplementary material, Table 1, runs 1–16). An overnight anaerobic culture in WMB was washed in 20 mM potassium phosphate buffer pH 7 (PB7), adjusted to an $OD_{650nm} = 1$ and used as an inoculum (1% v/v). Incubation was performed for 16 h at 37 °C in anaerobiosis (Generbox jars, BioMérieux SA, Marcy-l'Etoile, France, with AnaeroGenbags, Oxoid). The OD_{650nm} (BIO-RAD SmartSpec™Plus) and pH values (Double Pore Slim electrode, Hamilton Company, Reno, Nevada, USA) were measured and, only under the conditions in which the highest OD_{650nm} was observed, cells were enumerated on WMB agar (WMA, pH 6.8, 37 °C, 48 h) in anaerobiosis (Generbox jars, BioMérieux SA, Marcy-l'Etoile, France, with AnaeroGenbags, Oxoid). Biomass was measured by drying washed biomass (105 °C, 24 h). A standard curve relating OD_{650nm} and cell counts or cell dry weight (liner regression) was used to estimate the number of cells or biomass in all the other conditions. Eight further trials (runs 17–24, Supplementary material Table 1) were then carried out by varying yeast extract, tryptone, Tween 80 and Mn–Mg solution to validate the model. The cost of different media was estimated directly from the cost of the ingredients.

2.4. Kinetics of growth during pH controlled fermentation

The kinetics of growth of *L. rhamnosus* 64 was evaluated during batch fermentations in the optimized diluted whey permeate (diluted whey permeate with 2.5 g/L yeast extract, 2.5 g/L tryptone, 5 mL/L Mg–Mn solution) under anaerobic (AN, nitrogen 0.1 vol vol^{-1} min^{-1}) or aerobic (AE, air 0.1 vol vol^{-1} min^{-1}) conditions at 37 °C. Mg–Mn solution was composed of 2% (w/v) $MgSO_4$ and 0.4% (w/v) $MnSO_4$. Fermenters (1 L working volume, Applikon, Schiedam, the Netherlands) were inoculated (1% v/v) with an overnight anaerobic WMB pre-culture. Agitation was controlled using a magnetic stirrer (1 L bioreactor, 250 rpm, Heidolph MR Hei-Mix D), while pH was controlled (*ez*Control controller, Applikon) by automatic addition of sterile Na_2CO_3/NaOH (1:1 mix of a 4 eq/L solution of each base) to keep it at a value of 6. Growth was estimated by absorbance at 650 nm (OD_{650}) as described above; when needed the cell suspension was diluted with sterile medium to bring OD_{650} in the range of 0.05–0.700, and the dilution factor (D) was used to calculate D^*OD_{650} (DOD). Calibration curves relating cell counts or cell dry weight to DOD were estimated as described above.

The amount of alkali pumped in the fermenter was monitored at 30 min intervals using the dose monitoring function of the controller units (EZcontrol, Applikon, Schiedam NL). Data were transformed in ln (meq alkali/L) and plotted against the ln DOD values.

The growth curves of all fermentations (as estimated lnXX0 data from ln meq alkali/L) were modelled using the dynamic model of Baranyi and Roberts (1994) with DMFit v. 2.0 (Baranyi & Le Marc, 1996).

2.4.1. Chemical analyses

Lactose, galactose, D- and L-lactic acid and acetic acid were quantified using enzymatic kits (R-Biopharm AG, D-64293 Darmstadt, Germany).

2.5. Effect of exposure to mild stress on the response to heat and oxidative challenge

Tolerance to heat and oxidative challenge (HC and OC, respectively) was evaluated by exposing, for 15 min, exponential or stationary cell suspensions to 55 °C (HC) or H_2O_2 (OC) respectively. Prior to challenge, cultures were subjected (or not) to different mild stresses, directly in the fermenter. Stresses studied were: aerobic mild stress (AER, 1 h air 0.3 vol vol^{-1} min, at pH 6 and 37 °C); heat mild stress (HEAT, 1 h at pH 6 and 45 °C, anaerobiosis) and acid mild stress (ACID, 1 h at pH 5 and 37 °C, anaerobiosis). Control (untreated cells) and mild stress-treated cells were recovered from the fermenter and harvested by centrifugation at 12000× *g*, 5 min, washed twice in PB7 and resuspended in PB7 (to final $OD_{650} = 1.0$)

in pre-warmed (55 °C) PB7 (HC) or in pre-warmed (37 °C) PB7 containing 0.4 mmol/L H_2O_2 (OC). Cell counts were performed by pour plating in WMA pH 6.8 + 0.05% w/v cysteine and on WMA pH 5.5 (HCl addition), followed by incubation at 37 °C for 48 h in anaerobiosis. For a single fermentation, tolerance to HC and OC of control and acid-treated stationary phase cells was evaluated after the exposure of cell suspensions to heat or H_2O_2 for 0, 2, 5, 10, 15 and 30 min.

2.6. Selection of the matrix for spray drying

L. rhamnosus 64 biomass was obtained as described above in a 2 L fermenter (Sartorius Biostat A plus), inoculated (1% v/v) with an overnight anaerobic MRS broth pre-culture. As culture medium 9% (w/v) reconstituted cheese whey permeate (Arla Foods, Porteña, Córdoba, Argentina) supplemented with 2.5 g/L yeast extract, 2.5 g/L tryptone, 5 mL/L Mg–Mn solution was used. Fermentation was conducted in anaerobiosis with CO_2 flow (CO_2 0.2 L/min, 200 rpm stirring) maintaining pH at 6 by automatic addition of sterile Na_2CO_3/NaOH. Cells were harvested (6000 rpm, 15 min, 4 °C) and washed twice with PBS (pH 7.4). Cells were resuspended in (10% w/v) cheese whey (Arla Foods, Porteña, Córdoba, Argentina) plus 10% (w/v) starch (Glutal S.A., Buenos Aires, Argentina), or 10% (w/v) arabic gum (Gelfix, Buenos Aires, Argentina), or 10% (w/v) maltodextrin (Gelfix, Buenos Aires, Argentina) or 10% (w/v) WPC80 (Arla Foods, Porteña, Córdoba, Argentina). Cell suspensions were spray dried in a laboratory scale spray dryer (Buchi mini spray dryer model B290, Flawil, Switzerland). Drying conditions were: feeding rate: 270 mL h^{-1}, constant inlet air temperature: 140 °C, outlet temperature: 83 °C and air flux: 357 L h^{-1}. Cell viability was determined by plate counts (48 h, 37 °C, aerobiosis, MRS agar, Biokar, Beauvais, France) before and after spray drying and after 30, 90 and 120 days of storage of vacuum-sealed powders maintained at 5 °C in the dark.

2.7. Application of mild heat stress and spray drying in cheese whey-starch

Biomass of *L. rhamnosus* 64 was produced as indicated in the previous item. After biomass production, 1 L of culture was removed from the fermenter (control culture) and mild heat stress was applied (1 h, 45 °C, 200 rpm) to the remaining culture in the fermenter. Treated and control cells were harvested (6000 rpm, 15 min, 4 °C), washed twice with PBS (pH 7.4) and resuspended in cheese whey-starch solution (10%–10% w/v of each ingredient). Cell suspensions were spray dried as described above. Cell viability was determined (48 h, 37 °C, aerobiosis, WMA agar, Biokar, Beauvais, France) before and after spray drying. In another assay, biomass was produced in the same way in the fermenter, transferred to flasks (400 mL samples) and another set of mild stress treatments were applied: control (no treatment), high heat (52 °C, 15 min), low pH (60 min, pH 4 achieved by addition of 85% lactic acid, Tuteur S.A.C.I.F.I.A., Buenos Aires, Argentina) or combined high heat-low pH (52 °C, 15 min, pH 4). Treated and control cells were harvested (6000 rpm, 15 min, 4 °C), washed twice with PBS (pH 7.4), resuspended in cheese whey-starch solution (10%–10% w/v) and spray dried as described above. Cell viability was determined (48 h, 37 °C, aerobiosis, MRS agar, Biokar, Beauvais, France) before and after spray drying.

2.8. Statistical analysis

Statistical analysis and graphing were carried out using Systat 13 (Systat Inc. Chicago, IL). The results of the fractional factorial experiment were analysed using the General Linear Model Procedure of Systat 13.0 (Systat Inc., Chicago, IL). Both main factor effects and interactions were calculated. ANOVA and multiple mean comparisons (Tukey's HSD test) were used to analyse the results of the stress treatments performed at fixed time. The kinetics of inactivation was fitted using a Weibull model (Van Boekel, 2002).

3. Results

3.1. Biomass production in whey permeate

Growth of *L. rhamnosus* 64 in unsupplemented diluted whey permeate was poor, achieving 1–1.1 × 10^8 CFU/mL in stationary phase (1.3 log increases over the inoculum). A 2^{5-1} factorial experimental design was used to optimize the growth in diluted whey permeate (25 g/L lactose), using yeast extract, tryptone, Tween 80 and Mg–Mn solution as supplements (Table 1, supplementary material runs 1–16).

All factors had a significant effect on growth, either as main factors or in two factor interactions and the model had an adjusted $R^2 = 0.99$. Coefficient estimates and their significance are shown in Table 2 (supplementary material). The factors with the largest positive effect were Yeast extract and Tryptone, while the effect of sodium carbonate addition was negative. The interpretation of the effect of Tween 80 and Mg–Mn solutions was difficult due to factor interactions. Therefore, eight further trials were carried out (runs 17–24, Table 1, supplementary material) to validate the model and to design a minimum which would maximize growth while minimizing cost. The absolute deviation of experimental results in the validation set from predicted values was 2.86% (min 0.04, max 4.86%) thus, confirming the good predictive ability of the model. Run 20 provided the best results (9.2 × 10^8 CFU/mL and 1.3 g/L biomass at an estimated cost of 1.08 €/10^{12} cfu) and was used in all subsequent experiments.

3.2. Growth kinetics during pH controlled fermentation

The optimized medium was used at pH 6.5 controlled fermentations using Na_2CO_3/NaOH mixture as a neutralizing solution. These conditions were selected after preliminary experiments in which the effect of pH (6.0 or 6.5), neutralizing agent (NaOH, Na_2CO_3/NaOH or NH_4OH) or further addition of 2.5 g/L tryptone were compared (data not shown).

Fermentations were carried out in either anaerobic (N_2 flow; 8 replicates) or aerobic conditions (air flow, 2 replicates) and addition of base was monitored in all fermentations while growth kinetics was monitored (OD measure at 1 h intervals and cell counts and biomass determination at the end of the fermentation) in 2 fermentations under each condition. Preliminary trials showed that dose monitoring of alkali addition (at 30 min intervals, measured as logarithm of meq of base added) provided excellent estimates of biomass and log (viable counts) ($R^2 = 0.92$ in all fermentations); therefore, viable count values estimated from alkali addition were used as raw data to model the kinetics of growth using the Baranyi and Roberts (1994) D-model. This model provided a satisfactory reconstruction of experimental data ($R^2 > 0.99$). An example for biomass growth kinetics is shown in Fig. 1. Growth parameters and yields are shown in Table 1. The substrate was almost completely consumed (lactose <0.3 g/L). L-lactic acid was 94% of total lactic acid at the end of fermentation but it was only 70% in mid-exponential phase (data not shown). Acetic acid was always below the detection limit (0.02 g/L). Fermentations were highly reproducible (except for lag phase, which, not surprisingly, depended on the physiological state of the inoculum). Aerobic conditions resulted in a significantly ($p < 0.001$) decreased μ_{max} and biomass yield and lower final counts. The mean generation time in the exponential growth phase

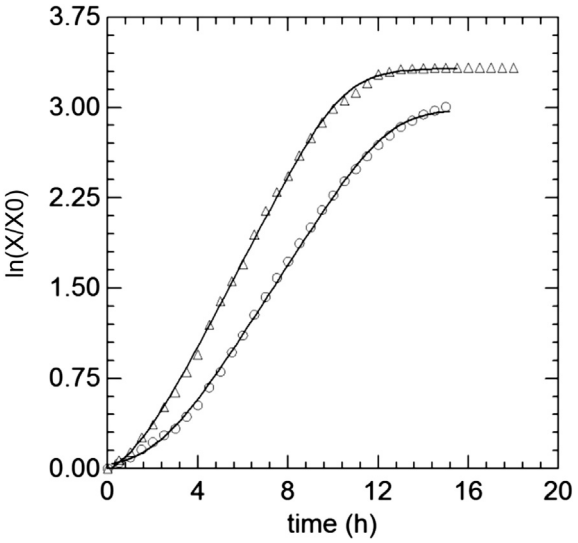

Fig. 1. Growth kinetics of *L. rhamnosus* 64 in optimized diluted whey permeate in aerobiosis (○) and anaerobiosis (Δ). X is the estimated cell dry weight whereas X0 is the cell dry weight at time 0.

was 1.95 h and 2.37 h in anaerobic and aerobic conditions, respectively.

3.3. Mild stresses and survival to heat and oxidative challenge

In order to evaluate the effect of aerobic or anaerobic growth on stress tolerance, early stationary phase cells grown under aerobic or anaerobic conditions were subjected to HC or OC for 15 min (Fig. 2). Survival to both challenges was significantly higher ($p = 0.02$) in the case of cells grown in anaerobiosis for both healthy cells (enumerated on WMA pH 5.5) and healthy + sub lethally injured cells (enumerated on WMA + cysteine). Survival to HC was significantly higher compared to survival to OC in both aerobiosis or anaerobiosis ($p < 0.001$). The fraction of damaged cells (0.39–0.41) was not significantly different among challenges and incubation conditions. Aerobic incubation significantly impaired tolerance to HC and OC in *L. rhamnosus* 64.

The effects of mild acid stress (pH 5,1 h) or mild heat stress (45 °C, 1 h) on the survival to HC and OC of exponential and stationary phase cells were evaluated (Fig. 3). The main effects (growth phase, mild stress/adaptation) and the interaction (growth phase × mild stress) all significantly ($p < 0.001$) affected the total number of surviving cells determined on WMA + cysteine. As expected, stationary phase cells were more tolerant than exponential phase cells. Heat stress of stationary phase cells resulted in the highest survival ($\log N/N_0 = -0.6$). Survival to HC of acid-treated cells was significantly higher than for non-treated stationary phase cells but not for exponential phase cells. As before, part of the population was sub-lethally damaged, and it was unable to form colonies on an acidified medium (WMA pH 5.5). Although the number of healthy survivors enumerated on WMA pH 5.5 was higher for heat-treated exponential phase cells compared to non-treated and acid-treated exponential phase cells ($p < 0.001$), the proportion of healthy survivors was not significantly different under any condition. OC was much more lethal than HC. The highest survival ($\log N/N_0 = -1.9$) was observed for acid-treated stationary phase cells, and the lowest ($\log N/N_0 = -3.1$) for heat-treated exponential phase cells. Both growth phase (stationary > exponential) and mild stress (acid > none > heat) had a significant effect ($p < 0.001$) on total survival to OC, while interaction was not significant. The proportion of damaged cells was significantly ($p < 0.001$) lower (14–18%) for heat and acid adapted exponential phase cells compared to non-treated cells, but it was in the same range as that observed for HC (40–60% in the case of all other treatments).

3.4. Kinetics of heat inactivation

Since kinetics of inactivation for non-spore forming bacteria are often non-linear, the kinetics of inactivation during heat or oxidative challenge of non-treated and acid-treated cells was fitted using a Weibull model: $\log(N/N_0) = -2.303 \cdot (t/\alpha)^{\beta}$. The alpha ($\alpha$) parameter is the characteristic inactivation time (the lower, the faster the decrease in survivors) whereas the beta (β) parameter is a shape parameter (<1 indicates an upward curvature, i.e. a tail; >1 indicates a downward curvature, i.e. a shoulder; 1 indicates that the model reduces to the classical exponential model for cell death). The time to reach d decimal (t_d) reduction can be calculated as: $t_d = (\alpha \cdot [-\ln(10^{-d})]^{\beta}$. For both heat and oxidative challenges the kinetics of inactivation was markedly non-linear, with an upward curvature (Fig. 4). The Weibull model (Table 2) provided a satisfactory fit for most inactivation curves (with R^2 ranging from 0.94 to 0.98 and residual mean square, RMS, ranging from 0.09 to 0.08). β values were <1 (because of the upward curvature) showing the existence of variability in tolerance within the population. Mild acid stress increased the resistance of both the total and uninjured population (as determined by higher α and t_{2D} values) to HC, but it had overall a minor effect on tolerance to OC in terms of parameters of the inactivation curve.

3.5. Spray drying and mild heat stress in cheese whey-starch

Different matrices were assessed in order to determine a suitable one for spray drying and storage of *L. rhamnosus* 64 as dehydrated culture. The highest survivals to spray drying were observed in the case of cheese whey-starch or cheese whey-arabic gum combinations, with reductions in logs of cell counts of ca. 0.5 (Figs. 5 and 6). Along storage, the smallest reductions in cell counts were observed for cheese whey-starch (ca. 0.6 log orders) or cheese whey-WPC80 (ca. 0.5 log orders) by day 90. By day 120, the smallest reductions were observed in cheese whey-WPC80 (ca. 0.5 log orders). When the strain was finally spray-dried, no significant differences were observed in the reduction in log orders of heat-treated cells (0.9 ± 0.1) compared to control cells (0.7 ± 0.2). In order to determine whether harsher conditions of heat or acid might enhance survival to drying, a heat treatment of 52 °C for 15 min, an acid treatment (pH 4, 60 min, 37 °C) or their

Table 1
Kinetic parameters for fermentations performed with *L. rhamnosus* 64 in a diluted whey permeate medium in anaerobiosis (AN, mean and standard error of 6 fermentations) and aerobiosis (AE, mean and standard error of 2 fermentations). Maximum specific growth rate (μ_{max}), duration of the lag phase (lag), percentage of substrate consumed (%), yields ($Y_{X/S}$ biomass yield, g/g substrate, $Y_{P/S}$ lactic acid yield, g/g substrate), final biomass (X_{max} and viable cell counts. With the exception of % substrate consumed and $Y_{P/S}$, the differences between AN and AE were always significant ($p < 0.001$).

Growth condition	μ_{max} (h^{-1})	lag (h^{-1})	%	$Y_{X/S}$	$Y_{P/S}$	X_{max}	log(cfu/ml)
AN	0.355 ± 0.004	2.56 ± 0.61	99.16 ± 0.04	0.11 ± 0.01	0.93 ± 0.01	3.05 ± 0.04	10.1 ± 0.0
AE	0.292 ± 0.004	2.29 ± 0.07	99.14 ± 0.03	0.08 ± 0.01	0.93 ± 0.01	2.82 ± 0.01	9.7 ± 0.2

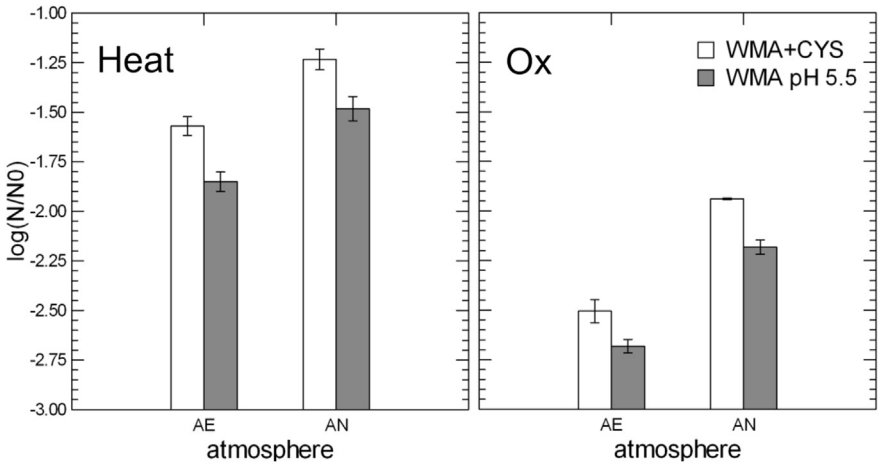

Fig. 2. Effect of heat (55 °C, 15 min) and oxidative challenge (0.04 mmol/L H_2O_2, 15 min, 37 °C) on the survival of early stationary phase cells *L. rhamnosus* 64 grown in optimized diluted whey permeate medium under aerobic (AE) or anaerobic (AN) conditions. After challenge, surviving cells were enumerated on WMA + cysteine (healthy + sublethally damaged cells) and on WMA pH 5.5 (undamaged cells only).

combination (52 °C, 15 min, pH 4) were applied to early stationary growth phase cells of *L. rhamnosus* 64 before its spray drying in cheese whey-starch. Higher cells deaths were observed after spray drying compared to the application of the mild stress previously studied. Additionally, low pH stress or combined low pH-heat stress induced significantly higher reductions in cell counts (Table 3).

4. Discussion

Food technologists are being challenged to produce probiotic cultures with high viability using low cost culture media and dehydration technologies in order to satisfy the increasing demand for probiotics and to cover the new food markets where these beneficial microbes are used (Muller et al. 2009). In a previous

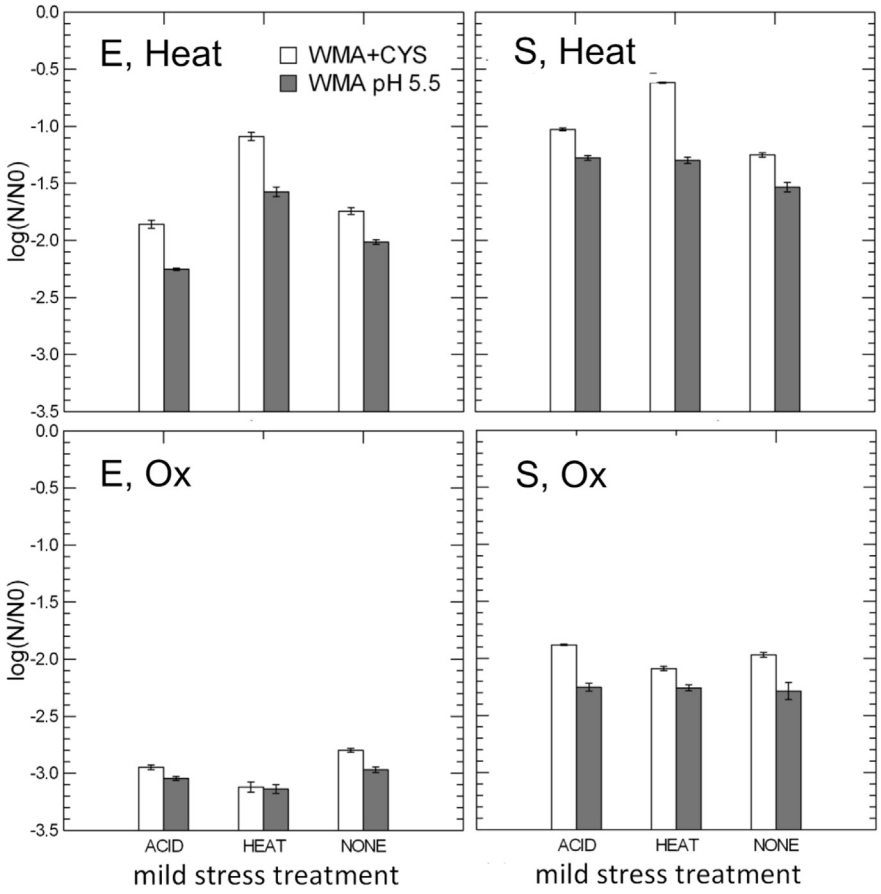

Fig. 3. Effect of heat (55 °C, 15 min) and oxidative challenge (0.04 mmol/L H_2O_2, 15 min, 37 °C) on the survival of exponential (E) or early stationary phase (S) cells of *L. rhamnosus* 64 grown in optimized diluted whey permeate medium under anaerobic conditions and submitted to mild acid (pH 5, 1 h) or mild heat stress treatment (45 °C, 1 h). After challenge, surviving cells were enumerated on WMA + cysteine (healthy + sublethally damaged cells) and on WMA pH 5.5 (undamaged cells only).

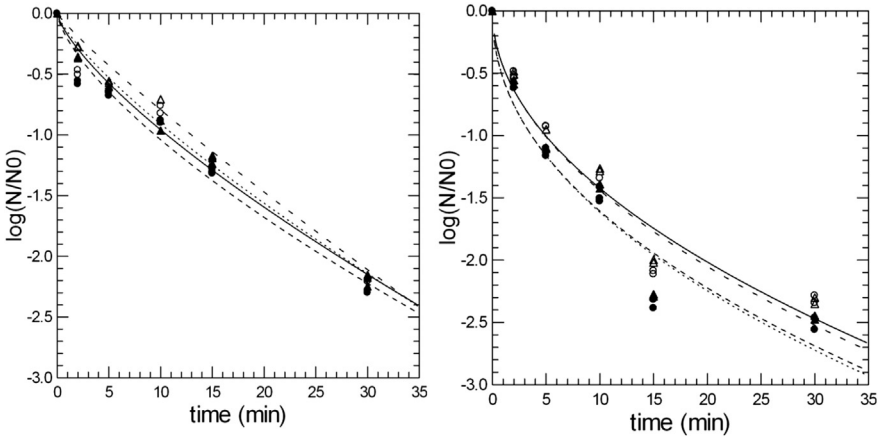

Fig. 4. Survival curve for early stationary phase cells of *L. rhamnosus* 64 grown in optimized diluted whey permeate medium during heat (left, 55 °C) and oxidative (37 °C, 0.04 mol/L H_2O_2) challenge. Circles: no previous mild stress; triangles: acid mild stress (pH 5 for 1 h). Empty symbols: total survivors (healthy + sublethally injured cells) enumerated on WMA + cys; closed symbols: healthy cells enumerated on WMA pH 5.5. The lines show the fit of the Weibull model.

work, unsupplemented cheese whey, whey permeate and ricotta whey were reported adequate for biomass production of *L. rhamnosus* 64, achieving a 2 log orders increase (1% v/v inoculum) after 24 hr of pH-free cultivation (counts ranged from 8.7 to 9.0 log CFU/mL). However, biological oxygen demand in spent medium was still high after *L. rhamnosus* 64 growth, indicating perhaps the presence of non-consumed nutrients and excreted metabolites (lactate for instance) rich in carbon as energy source (Lavari et al., 2014). Additionally, multiple possibilities are now available for further cheese whey valorization (Prazeres et al., 2012) whereas whey permeate was less exploited, compared to cheese whey, for its valorization in relation to biomass production of lactic acid bacteria (Christopherson & Zottola, 1989; Parente & Zottola, 1991) or products derived from their metabolisms such as nisin (Desjardins, Meghrous, & Lacroix, 2001), exopolysaccharides (Macedo, Lacroix, Gardner, & Champagne, 2002) or lactic acid (Fitzpatrick & O'Keeffe, 2001; Tuli, Sethi, Khanna, & Marwaha, 1985). In this context, we decided to explore the use of whey for enhanced biomass production of *Lactobacillus rhamosus* 64. Unsupplemmented diluted permeate resulted in 1.3 log orders growth in uncontrolled (free) pH fermentation, compared to the ca. 2 log orders previously observed (Lavari et al., 2014) in non-diluted whey permeate (7.47 g/100 mL of lactose). The 2^{5-1} fractional factorial experiment allowed an ca. 1 log order increase in cell counts being Yeast extract and Tryptone the factors with the largest positive effect on growth. Nitrogen sources as the ones used are known growth promoting ingredients for LAB (Burns et al., 2008; Macedo et al., 2002).

The supplemented diluted whey permeate was used to study the kinetics of growth during pH controlled fermentation under aerobiosis and anaerobiosis, the latter being the most suitable condition (cell counts ca. 10^{10}). Additionally, remaining lactose was very low. The ability of lactobacilli to use oxygen as a terminal electron acceptor in an electron transport chain, or to use it via the pyruvate oxidase/acetate kinase pathway varies (Liu, 2003; Pedersen, Gaudu, Lechardeur, Petit, & Gruss, 2012; Zotta, Guidone, Ianniello, Ricciardi, & Parente, 2013; Zotta et al., 2014) and although aerobic growth may sometimes improve growth yield and oxidative stress tolerance, the effect is strain dependent.

The effect of aerobic vs. anaerobic incubation was also studied in relation to the resistance to OC and HC (Fig. 2) of *L. rhamnosus* 64 harvested in the stationary growth phase. OC, HC and osmotic challenge are the main stress factors during spray drying (Peighambardoust, Tafti, & Hejazi, 2011). Studying OC and HC was a simplified way to separately analyse the responses to stresses found by lactobacilli during spray drying. *L. rhamnosus* 64 was more sensitive to OC than HC, under the conditions assessed, regardless of the aerobic or anaerobic biomass production. When it comes to aeration conditions, anaerobic incubation rendered cells more tolerant to both HC and OC. These results are contrary to those reported for *Lactobacillus plantarum* WCFS1 (Zotta et al., 2012). Strain-dependency of response to different stress has been reported for *L. plantarum* (Parente et al., 2010; Zotta et al., 2013) and *Lactobacillus casei* (Zotta et al., 2014). Guilbaud, Zagorec, Chaillou, and Champomier-Vergès (2012) working with several *Lactobacillus sakei* strains reported that resistance to oxidative stress was extremely variable even within a single species. Anaerobic fermentation was then chosen for further studies.

Table 2
Estimated parameters (estimate ± asymptotic standard error) of the Weibull model for survival after heat (H) and oxidative (O) challenge of stationary phase cells of *L. rhamnosus* 64 grown in optimized diluted whey permeate medium and subjected (or not: none) to mild acid stress (1 h, pH 5). Cell counts were performed on WMA + cys (injured + healthy cells) and WMA pH 5.5 (healthy cells only). Residual mean square (RMS), R^2 and time to reduce the population by 99% (t_{2D}, min) are indicated. Only significant digits are shown.

Cell count on	Mild stress	Challenge	Alpha	Beta	RMS	R^2	t_{2D}
WMA + cys	None	H	3.4 ± 0.5	0.74 ± 0.05	0.014	0.98	27.2
WMA + cys	Acid	H	5.08 ± 0.04	0.89 ± 0.05	0.009	0.98	28.3
WMA pH5.5	None	H	2.8 ± 0.4	0.69 ± 0.05	0.017	0.97	25.8
WMA pH5.5	Acid	H	3.0 ± 0.3	0.78 ± 0.04	0.080	0.99	27.5
WMA + cys	None	O	0.9 ± 0.3	0.50 ± 0.06	0.035	0.96	19.7
WMA + cys	Acid	O	1.0 ± 0.3	0.51 ± 0.05	0.035	0.95	19.0
WMA pH5.5	None	O	0.6 ± 0.2	0.47 ± 0.06	0.047	0.95	16.0
WMA pH5.5	Acid	O	0.6 ± 0.3	0.48 ± 0.06	0.059	0.94	15.7

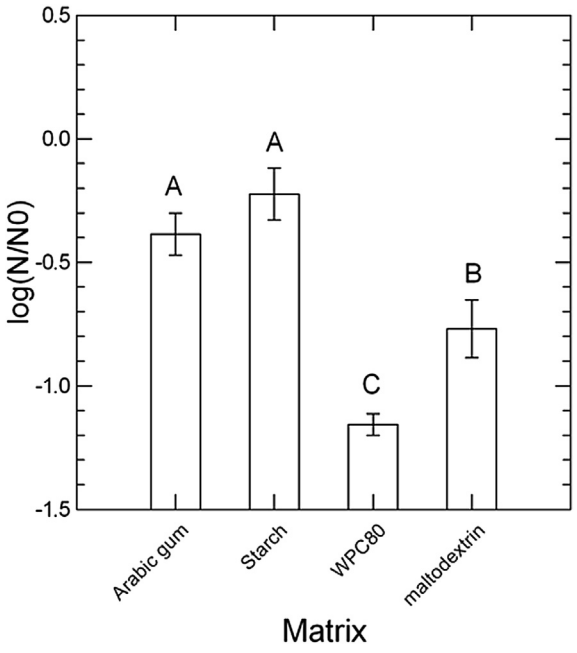

Fig. 5. Effect of spray drying of early stationary phase cells of *L. rhamnosus* 64 in 10% (w/v) cheese whey combined with 10% (w/v) arabic gum, starch, WPC80 or maltodextrin on survival to drying. Arithmetic meas with standard errors. Bars with the same letter are not significantly different (Tukey's HSD test $p <= 0.05$).

Mild heat and acid stress were applied to exponential and stationary cells before OC and HC (Fig. 3) in order to study resistance to these stress factors aiming at enhanced survival to spray drying. Cells harvested in the stationary growth phase were more resistant to both challenges. During growth in batch cultures, LAB are exposed to increasing levels of different stress factors. These factors by themselves or in combination can influence their growth and

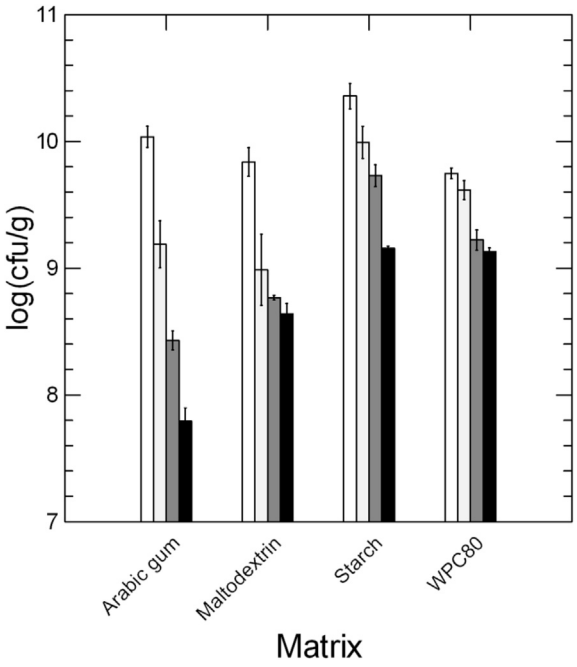

Fig. 6. Counts of *L. rhamnosus* 64 spray dried in 10% (w/v) cheese whey combined with 10% (w/v) arabic gum, starch, WPC80 or maltodextrin at time 0 (□), 30 (▒), 60 (▓) and 120 (■) of storage at 5 °C.

Table 3
Effect of heat (52 °C, 15 min), low pH (pH 4, 60 min, 37 °C) or the combination heat-low pH (52 °C, 15 min, pH 4) on the survival of *L. rhamnosus* 64 to spray drying in cheese whey-starch (10%–10% w/v).

Cell decay (difference in log orders CFU mL^{-1} before and after spray drying)			
Control	Heat	Low pH	Heat-low pH
1.21 ± 0.18^{a}	1.73 ± 0.11^{b}	2.17 ± 1.72^{c}	2.05 ± 0.42^{c}

[a,b,c] Mean ± standard deviation. Values with different superscript are significantly different ($p < 0.05$).

functionality (Németh, Adányi, Halász, Váradi, & Szendrő, 2007). The cytoplasmatic synthesis of molecular chaperones (Sugimoto, Abdullah-Al-Mahin, & Sonomoto, 2008) can render stationary phase cells, generally but not always, more tolerant to further stress than cells harvested in the exponential growth. For example, in a previous work, bifidobacteria harvested in the exponential or stationary growth phase showed differences in their resistance to simulated gastric digestion (Vinderola et al., 2012). However, many other studies showed differences in the functionality of probiotic cells when harvested at different time points along the growth curve (Maassen, Boersma, van Holten-Neelen, Claassen, & Laman, 2003; Sashihara, Sueki, Furuichi, & Ikegami, 2007). As for cells harvested in the stationary phase, mild heat stress was more effective to partially prevent the detrimental damage caused by heat challenge. This is in coincidence with the generally accepted fact that pre-exposure to sublethal levels of a stress factor induces an enhanced resistance against subsequent exposure to higher levels of the same stress (Bunning, Crawford, Tierney, & Peeler, 1990; O'Driscoll, Gahan, & Hill, 1996).

Different survival capacities were observed for each mixture of ingredients used (starch, arabic gum, WPC80 or maltodextrin), being starch and arabic gum the most suitable ones (Figs. 5 and 6). Intrinsic differences in glass transition temperatures of the ingredients used might be related to the different protection capacity towards cells viability, as indicated by Carvalho et al. (2004) and Aschenbrenner, Kulozik, and Foerst (2012) when studying the stability of dehydrated LAB.

The results obtained so far and some practical issues to be taken into account at the industrial level (easiness of management of ingredients, solubility and cost, among others) that may determine the feasibility of scaling-up, were considered for choosing a mild stress treatment and a matrix for spray drying, aiming at enhancing survival to spray drying. Considering together the survival to drying and along storage for 120 days and the lower cost of starch compared to WPC80, we decided to use cheese whey-starch, with a previous application (or not) of a mild heat stress (45 °C for 1 h). Heat stress was chosen over acid stress since it yielded reasonably similar survival rates to heat challenge (Fig. 3). Additionally, at a higher scale, the application of a mild heat stress is technologically easier than the acidification and neutralization of the cell suspension before spray drying, as it is not recommended to spray dry cells at low pH since medium becomes sticky (Bylund, 1995). The mild heat stress applied (45 °C, 1 h) or the harsher conditions further studied (52 °C for 15 min, pH 4) for 60 min at 37 °C, or their combination (52 °C, 15 min, pH 4) were not effective for our aims.

5. Conclusion

Supplementation of diluted whey permeate allowed the production of ca. 10^{10} CFU/mL of *L. rhamnosus* 64 under pH controlled fermentation in anaerobiosis. Under all conditions assessed, OC was more detrimental against cell viability compared to HC. Anaerobic incubation, cell harvesting at the stationary growth phase and mild

heat stress were more effective for reducing cell death towards heat challenge, whereas cheese whey-starch was effective towards cell protection to spray drying and storage. However, no effects on cell survival to spray drying were observed when the conditions assessed in this study were used to increase resistance to spray drying in cheese whey-starch solution. Further conditions will be tested in the future to determine their protective effect against cell inactivation during spray drying or/and along storage of the powders obtained.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2015.03.066.

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