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Specific Gaseous Conditions Significantly Improve *Lactobacillus casei* and *Escherichia coli* Survival to Freeze Drying and Rehydration

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Abstract

Background and objective: Presence of oxygen during production and rehydration of freeze-dried starters and probiotics can decrease viability of the bacteria. Indeed, removal of water from cells during freeze-drying can promote dysfunction in anti-oxidative mechanisms, resulting in oxidative stress by accumulation of reactive oxygen species. The aim of this study was to show how atmospheric or less oxidative gaseous conditions affect bacterial survival to freeze-drying and rehydration of two strains, including *Lactobacillus casei*, a widely used bacteria in biotechnology, and *Escherichia coli*, a laboratory model bacteria.

Material and methods: *Lactobacillus casei* ATCC 334 and *Escherichia coli* K12 were freeze dried for 24h in 5% sucrose (m v^{-1}). Two gaseous conditions (an oxygen-free gas and atmospheric air) were used during various steps of the process, including bacterial cultivation, mixing of the bacteria with the protectant and rehydration. Oxygen-free gas condition was obtained with an oxygen-free gas, composed of nitrogen, hydrogen and carbon dioxide ($N_2H_2CO_2$) and an anaerobic chamber.

Results and conclusion: Gaseous conditions included significant effects on bacterial survival rates ($P<0.001$ for *Lactobacillus casei* and *Escherichia coli*). Interestingly, for both bacteria, the optimal combination was atmospheric air during mixing of the bacteria with the lyoprotectant ($P<0.001$ for *Lactobacillus casei* and *Escherichia coli*) and $N_2H_2CO_2$ during rehydration ($P<0.001$ for *Lactobacillus casei* and $P<0.05$ for *Escherichia coli*). Management of gaseous conditions during a freeze-drying process and rehydration (atmospheric air during mixing of the bacteria with lyoprotectant and oxygen-free gas during rehydration) enhances survival of the bacteria by preserving them from oxidative stress.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Stabilization of bacteria through drying is indispensable to preserve viability over a long term until the bacteria are used in food industries as starters or probiotics. Removal of the intracellular water from bacteria until a low water activity level ($a_w \leq 0.3$) achieved can result in biomass stabilization by slowing or aborting cell metabolic activities [1]. Freeze drying is the most used technology for the production of probiotics and starters in dry forms because this process leads to appropriate survival rates [2,3]. The entire production process of freeze-dried bacterial biomass generally includes microorganism culture and cell preparation, freeze-drying, packaging, storage and rehydration. Throughout the process, technological constraints (temperature, acid, osmotic, starvation and oxidative stresses) can affect the bacterial growth rate and survival [4], mainly causing permeabilization of the plasma membrane [5]. In presence of oxygen, bacteria generate reactive oxygen species (ROS). Of these ROS, free hydroxyl radicals are especially aggressive to DNA as well as membrane phospholipids and proteins [6]. Oxidation occurs during freeze drying, exactly during the freezing step, due to ROS accumulation in cells [7-9]. Fredrickson et al. have

suggested that protein oxidation due to ROS accumulation is the major cause of desiccation damages in bacterial cells [10]. To preserve the bacterial viability through these processes, protective strategies have been used against oxidative stresses. Use of oxygen-free gases to prevent oxidative damages in stabilization of bacteria seems to be a promising strategy. Studies have used a gaseous mixture of nitrogen, hydrogen and carbon dioxide ($N_2H_2CO_2$) during biomass production of the oxygen-sensitive bacteria [11-13]. However, this mixture has never been used in production and rehydration of freeze-dried bacteria. The gaseous mixture of $N_2H_2CO_2$ is an efficient reducing mixture close to gaseous condition of the large intestine, the natural habitat of most common commensal bacteria used as starters or probiotics. Therefore, the aim of this study was to assess benefits of using mimetic intestinal gaseous conditions to protect bacteria from oxidative stress during various steps of bacterial freeze-dried biomass production process.

2. Materials and methods

1.1. Production process of the freeze-dried bacterial biomass

Effectiveness of the protection strategy was assessed by comparison of the bacterial survival rates in presence of two gases respectively simulating intestinal gas and atmospheric air during various steps of the process. The process steps included culture of the microorganisms to produce the initial biomass, preparation of the cells to freeze drying, freeze-drying under vacuum and rehydration before the viability assessment. Atmospheric air (A) or mimetic intestinal gas (I) were used during various steps of the entire process. The mimetic intestinal gaseous condition was achieved using a mixture of $N_2H_2CO_2$ (85, 5 and 10%) provided by Air Liquide (Paris, France) and Sheldon Bactron II Anaerobic Chamber (Sheldon, Cornelius, USA) as described previously [14].

1.1.1. Bacteria and cultivation conditions

Obligate aerobe and anaerobe bacteria were excluded from this study; hence, facultative anaerobe and oxygen-tolerant bacteria were used. *Escherichia* (*E.*) *coli* K12 ATCC 10798 (facultative anaerobe) and *Lactobacillus* (*L.*) *casei* ATCC 334 (oxygen tolerant anaerobe) were chosen. *E. coli* is a laboratory model bacteria and *L. casei* is a lactic acid bacteria (LAB) with important uses in industrial and biotechnological fields. The *E. coli* strain was inoculated (A/I) onto lysogeny broth (LB) agar (15 g l^{-1}) (Sigma-Aldrich, USA) and *L. casei* (A/I) was inoculated onto de Man, Rogosa and Sharpe (MRS) agar (15 g l^{-1}) (Biokar Diagnostics, France) and incubated (A/I) at 37°C for 24 h. One colony of each bacteria was inoculated (A/I) into 10 ml of LB for *E. coli* and 10 ml of MRS for *L. casei* and incubated (A/I) at 37°C for 16 h. Then, 10 ml of the final cultures were inoculated at 1% v v^{-1} (A/I) to subcultures and incubated (A/I) at 37°C for 16 h. For the I condition, media inoculation and culture incubation were carried out in an anaerobic chamber and media were degassed through gas bubbling with $N_2H_2CO_2$ (85, 5 and 10%) for 4 h by passaging through a syringe filter (0.22 µm). For the A condition, culture inoculation and incubation were not carried out in an anaerobic chamber and media were not degassed.

1.1.2. Preparation of cells before freeze drying

Preparation of cells was carried out with harvesting by centrifugation and mixing with drying protectants. Cell suspensions were washed using centrifugation (2,000× g, 5 min, 25°C) with non-degassed phosphate buffered saline (PBS) (Sigma-Aldrich, USA) for the A condition or degassed PBS for the I condition. Cells were suspended in non-degassed PBS (A condition) or degassed PBS (I condition), both supplemented with 5% of sucrose (m v^{-1}) (Sigma-Aldrich, USA). In this study, sucrose was used as lyoprotectant since this sugar is currently used as standard in bacterial freeze drying, as same as trehalose [15]. Briefly, 1 ml of each bacterial suspension (bacterial biomass and lyoprotectant) was transferred into 5-ml vials (A/I) and vials were transferred to hermetic plastic boxes (A/I). For the I condition, PBS and PBS supplemented with sucrose were degassed using a method previously described. Preparation was carried out in an anaerobic chamber. For the A condition, media were not degassed and the preparation were not carried out in the anaerobic chamber.

1.1.3. Freeze drying

Closed hermetic plastic boxes containing vials were frozen at -80°C with a slope of -2°C min^{-1}. The bacteria were lyophilized for 20 h using single-chamber freeze-dryer (FreeZone 18-Liter Console Freeze Dry System with Stoppering Tray Dryer, Purge Valve and PTFE-Coated Collector, Labconco, Kansas City, USA) to achieve a final a_w of 0.25. Temperature and pressure of the condenser were set as -55°C and 5 Pa, respectively. Water activity was calculated using Aqualab CX-2 Osmometer (Decagon Devices, Pullman, WA, USA).

1.1.4. Rehydration

Rehydration was carried out at room temperature to recover *E. coli* and *L. casei* with 1 ml of A or I culture media (LB and MRS for *E. coli* and *L. casei*, respectively).

1.2. Effects of gaseous conditions

1.2.1. Bacterial enumeration

Bacteria were enumerated after the upstream steps of freeze-drying and at the end of the process. Viable cells were enumerated using traditional plating method (CFU ml^{-1}). Plates were incubated at 37°C for 35 h.

1.2.2. Gaseous conditions during upstream steps of the freeze-drying process

Atmospheric air and mimetic intestinal gaseous conditions were compared with each other, regarding bacterial production and recovery after centrifugation and mixing with drying protectants. Two gaseous conditions (A/I) were used during cultures as well as preparations of cells. The *E. coli* and *L. casei* concentrations (CFU ml^{-1}) were compared within four possible combinations of the gaseous conditions. These combinations included AA, AI, IA and II. The first letter corresponded to gaseous conditions during the culture and the second letter corresponded to gaseous conditions during the preparation of cells.

1.2.3. Use of various gaseous conditions during the entire freeze-drying process

Atmospheric air and mimetic intestinal gaseous conditions were compared with each other to improve bacterial survival to freeze-drying and rehydration. As described for upstream steps of freeze-drying, two gaseous conditions were used for the cultivation, preparation of cells and rehydration. As freeze-drying was carried out in a vacuum, no gaseous conditions were used for this step. Based on *E. coli* and *L. casei* concentrations after the upstream steps of freeze-drying and those at the end of the entire process, the bacterial survival to freeze-drying were expressed as percentages. Therefore, *E. coli* and *L. casei* survival rates were compared with each other within eight possible combinations of the gaseous conditions, including AAA, AAI, AII, AIA, IAI, IAA, IIA and III. The first letter corresponded to gaseous conditions used during cultivation, the second letter corresponded to gaseous conditions during preparation of the cells and the third letter corresponded to gaseous conditions during rehydration.

1.3. Statistical analysis

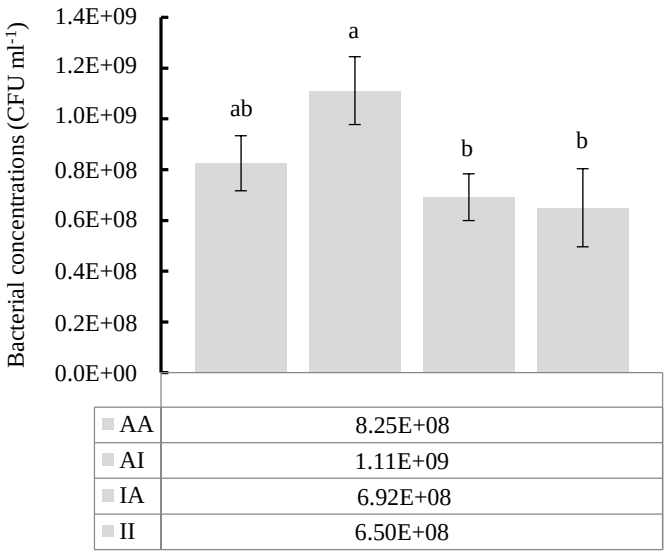
All experiments were carried out in completely independent triplicates (n = 3). The Analysis of Variance was used to investigate effects of gaseous conditions on bacterial concentrations and survival rates ($P \leq 0.05$). Tukey's Honest Significant Difference test was used if differences were significant with Analysis of Variance. Significant differences between the bacterial concentrations and survival rates were shown with different letters in the figures. One-sample t-test was used to compare mean values with specified values ($P \leq 0.05$). The R Software v.3.3.2 (R Development Core Team, 2008) was used to statistically analyze data.

3. Results and discussion

3.1. Bacteria production depends on gaseous conditions during upstream steps of the freeze-drying

Concentrations of *E. coli* (Fig. 1a) and *L. casei* (Fig. 1b) were assessed after cultivation and preparation and before Freeze-drying at two variously gaseous conditions of atmospheric air (A) and mimetic intestinal gas (I).

a) *Escherichia coli*



b) *Lactobacillus casei*

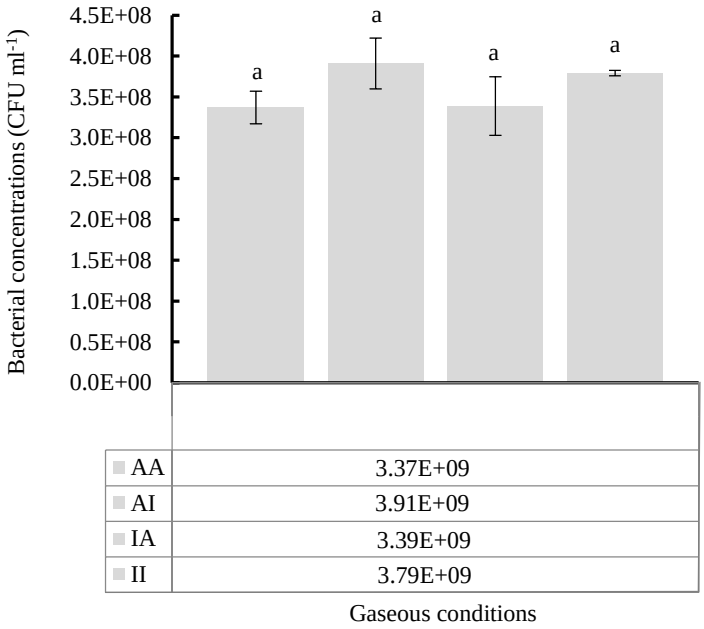


Figure 1. Effects of gaseous conditions on *Escherichia coli* (a) and *Lactobacillus casei* (b) mean cell concentrations (CFU ml^{-1}) during upstream steps of the freeze-drying. A (atmospheric air) and I (mimetic intestinal gaseous condition- $N_2H_2CO_2$) represent gaseous conditions for cultures and preparations of cells before freeze-drying. Different letters indicate significant differences between the bacterial concentrations (Tukey's Honest Significant Difference test, $P \leq 0.05$).

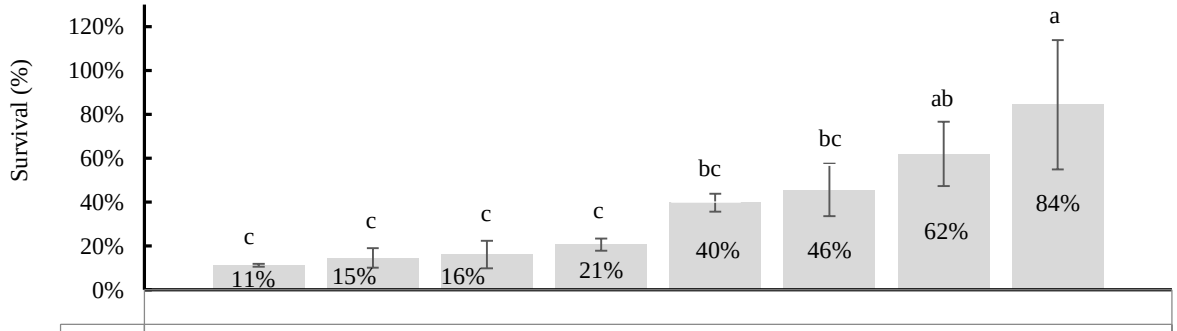
For *E. coli*, significant effects of gaseous conditions on bacterial counts before freeze-drying were reported ($P<0.05$). Gaseous conditions during cultivation included significant effects on *E. coli* cell concentrations ($P<0.01$), whereas gaseous conditions during preparation did not include any effects ($P=0.20$) (Fig. 1a). A greater biomass (mean bacterial concentration of 9.7×10^8 CFU ml^{-1} $\pm 2.1 \times 10^8$) was achieved after cultivation under atmospheric air conditions, compared to that achieved after cultivation under mimetic intestinal gaseous conditions (mean bacterial concentration of 6.7×10^8 CFU ml^{-1} $\pm 1.4 \times 10^8$) (Fig. 1a). As a facultative anaerobe, *E. coli* can produce energy and biomass with or without oxygen. However, aerobic respiration produces more ATP from 1 M of glucose, compared to that fermentation does. For *L. casei*, no significant differences were seen between the bacterial counts within the four possible combinations of gaseous conditions ($P=0.16$) (Fig. 1b). In fact, *L. casei* is an oxygen-tolerant anaerobe and a facultative hetero-fermentative species that reveals that *L. casei* grew fermentatively, resulting in a similar biomass production with air or mimetic intestinal gaseous conditions (Fig. 1b).

3.2. Bacteria survival to entire process depends on gaseous conditions

In fact, *E. coli* and *L. casei* were submitted to four successive steps of the entire process such as cultivation, preparation, freeze-drying and rehydration. Since freeze-drying was carried out under vacuum, gaseous conditions were modulated in three steps, corresponding to eight possible combinations of the gaseous conditions. Results are presented in Figs. 2a and 2b for *E. coli* and *L. casei*, respectively. Gaseous conditions significantly affected the bacterial survival to the process ($P<0.001$ for *E. coli* and *L. casei*). Indeed, survival rates ranged from 11% ±1 to 84% ±24 for *E. coli* and from 20% ±4 to 111% ±13 for *L. casei*. For *L. casei* with AAI and IAI, the survival rates were not significantly different from 100% as revealed by one-sample *t*-test. Hence, in AAI and IAI, freeze-drying and rehydration processes did not affect the *L. casei* viability. Similarly, gaseous conditions had a significant effects on the final viable bacterial concentrations (e.g. after freeze-drying and rehydration) of *E. coli* ($P<0.001$) and *L. casei* ($P<0.001$), as shown in Fig. S1. More generally, results showed that modification of gaseous conditions during cultivation did not include significant effects on the bacterial survival to the entire process (Fig. 2) ($P = 0.22$ for *E. coli* and $P=0.82$ for *L. casei*). Furthermore, gaseous conditions during cultivation did not include significant effects on the final viable bacterial concentrations (Fig. S1) ($P=0.67$ for *E. coli* and $P=0.91$ for *L. casei*). Previous studies on *E. coli* have shown that gaseous conditions during cultivation can affect the bacterial cell concentration before freeze-drying (Fig. 1a). These controversial results could be explained by the fact that two various but linked parameters were compared. These included biomass production after cultivation and biomass survival to freeze-drying and rehydration. It means that the low *E. coli* count due to the I condition after upstream steps of the freeze-drying was largely compensated by the *E. coli* high survival rate to downstream processes with the I condition. Comparison of the Figs. 2 with S1 verifies these results since the gaseous conditions similarly affects the final viable bacterial concentrations and the bacterial survival to freeze-drying and rehydration.

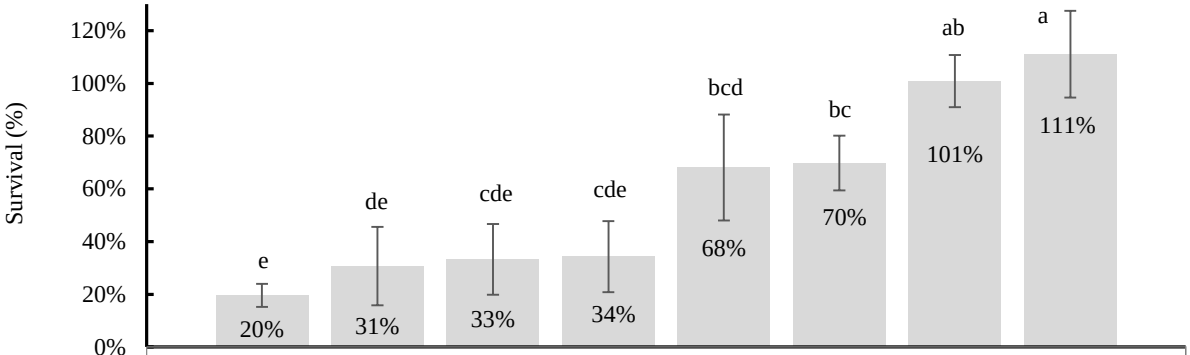
Figures 2 and S1 demonstrate that modification of gaseous conditions during preparation of the cells before freeze-drying significantly affected the bacterial survival to the entire process ($P<0.001$ for *E. coli* and *L. casei*) and the final viable bacterial concentrations ($P<0.001$ for *E. coli* and *L. casei*). A common feature of the low survival rates and the low final viable bacterial concentrations included preparation of the cells under mimetic intestinal gaseous conditions. Hence, the survival rates were below 35% for both bacteria. A common feature of the high survival rates and the high final viable bacterial concentrations included preparation of the cells under atmospheric air conditions. Preparation of the cells under atmospheric air conditions significantly improved the bacterial survival rates ($P<0.001$ for *E. coli* and *L. casei*), compared to the mimetic intestinal gaseous conditions. Selection of the atmospheric air instead of the mimetic intestinal gaseous condition in the preparation step resulted in significant increases in survival rates of more than 40 and 50% for *E. coli* and *L. casei*, respectively. For *E. coli*, preparation of cells under mimetic intestinal conditions resulted in a mean final viable bacterial concentration of 1.3×10^8 CFU ml^{-1} $\pm 5.8 \times 10^7$ (Fig. S1a) while preparation of the cells in atmospheric air resulted in a mean final viable bacterial concentration of 4.3×10^8 CFU ml^{-1} $\pm 1.5 \times 10^8$ (Fig. S1a). For *L. casei*, preparation of cells under mimetic intestinal conditions resulted in a mean final viable bacteria concentration of 1.1×10^9 CFU ml^{-1} $\pm 4.7 \times 10^8$ (Fig. S1b) while preparation of the cells under atmospheric air resulted in a mean final viable bacteria concentration of 3.0×10^9 CFU ml^{-1} $\pm 9.2 \times 10^8$ (Fig. S1b). Therefore, use of the atmospheric air in preparation steps resulted in a mean final viable bacterial concentration three times higher than that use of mimetic intestinal gaseous condition did.

a) *Escherichia coli*



AIA	11%
III	15%
IIA	16%
AII	21%
AAA	40%
IAA	46%
AAI	62%
IAI	84%

b) *Lactobacillus casei*

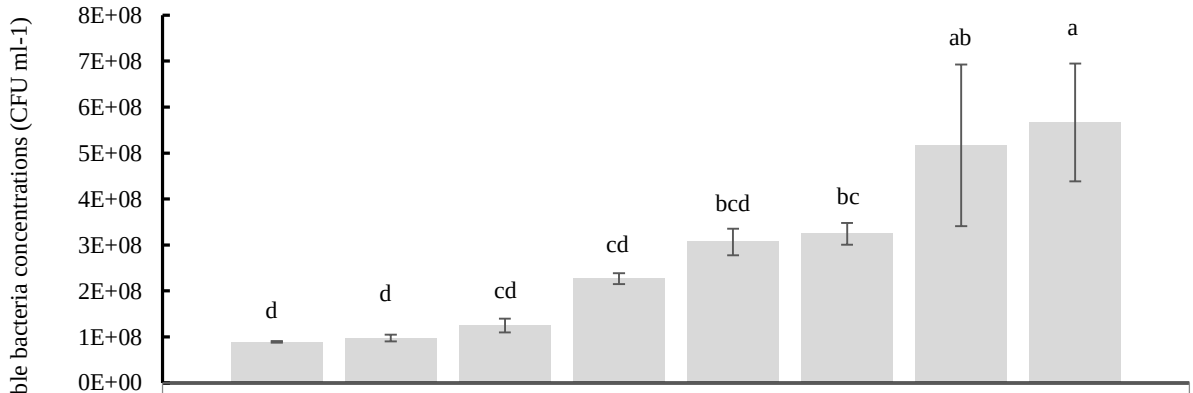


IIA	20%
III	31%
AIA	33%
AII	34%
AAA	68%
IAA	70%
AAI	101%
IAI	111%

Gaseous conditions

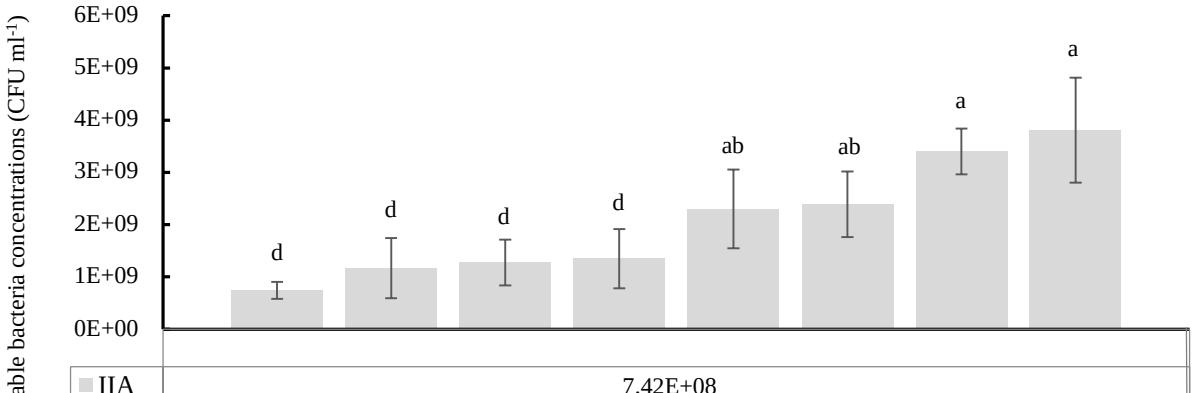
Figure 2. Effects of gaseous conditions on *Escherichia coli* (a) and *Lactobacillus casei* (b) survival (%) to freeze-drying and rehydration during the whole process (production of freeze-dried bacterial biomass and rehydration). A (atmospheric air) and I (mimetic intestinal gaseous condition - $N_2H_2CO_2$) represent gas conditions during cultures, preparations of the cells before freeze-drying and rehydration (freeze-drying was carried out in vacuum). Different letters indicate significant differences between the bacterial survival rates (Tukey's Honest Significant Difference test, $P \leq 0.05$).

a) *Escherichia coli*



III	8.92E+07
IIA	9.75E+07
AIA	1.25E+08
AII	2.27E+08
IAA	3.07E+08
AAA	3.24E+08
AAI	5.17E+08
IAI	5.67E+08

b) *Lactobacillus casei*



IIA	7.42E+08
III	1.17E+09
AIA	1.28E+09
AII	1.35E+09
AAA	2.30E+09
IAA	2.39E+09
AAI	3.40E+09
IAI	3.81E+09

Gaseous conditions

Figure S1. Effects of gaseous conditions on final *Escherichia coli* (a) and *Lactobacillus casei* (b) concentrations (CFU ml^{-1}) during the whole process (production of freeze-dried bacterial biomass and rehydration). A (atmospheric air) and I (mimetic intestinal gaseous condition-$N_2H_2CO_2$) represent gas conditions during cultures, preparations of the cells before freeze-drying and rehydration (freeze-drying was carried out in vacuum). Different letters indicate significant differences between the bacterial concentrations (Tukey's Honest Significant Difference test, $P \leq 0.05$).

Key operating parameters have been identified that affect survival of bacteria during cell preparation before freeze-drying. These include harvest [16], centrifugation (time and speed) [17], cell wash [18], cell concentration in freezing media [19], cryoprotectants (nature of the protective substance and concentration) [20] and pre-stresses [21]. Results of the present study have shown that use of atmospheric air during cell preparation can significantly improve viability of the facultative anaerobic and oxygen-tolerant bacteria during freeze-drying processes. Moreover, results have demonstrated that when cells are prepared with atmospheric air, rehydration with mimetic intestinal gaseous condition significantly improves the bacterial survival to entire process ($P<0.05$ for *E. coli* and $P<0.001$ for *L. casei*) and the final viable bacterial concentrations ($P< 0.01$ for *E. coli* and *L. casei*) (Figs. 2 and S1). Use of mimetic intestinal gaseous conditions instead of atmospheric air for rehydration provided significant increased survival rates of more than 20 and 30% for *E. coli* and *L. casei* respectively. For *E. coli*, preparation and rehydration of cells under atmospheric air conditions resulted in a mean final viable bacterial concentration of 3.1×10^8 CFU ml^{-1} $\pm 2.5 \times 10^7$ (Fig. S1a), while preparation of cells in atmospheric air and rehydration in mimetic intestinal gaseous condition resulted in a mean final viable bacterial concentration of 5.4×10^8 CFU ml^{-1} $\pm 1.4 \times 10^8$ (Fig. S1a). For *L. casei*, preparation and rehydration of cells in atmospheric air conditions resulted in a mean final viable bacterial concentration of 2.3×10^9 CFU ml^{-1} $\pm 6.2 \times 10^8$ (Fig. S1b), while preparation of cells in atmospheric air and rehydration in mimetic intestinal gaseous condition resulted in a mean final viable bacterial concentration of 3.6×10^9 CFU ml^{-1} $\pm 7.3 \times 10^8$ (Fig. S1b). Therefore, use of atmospheric air in preparation step and mimetic intestinal gas in rehydration step resulted in a mean final viable bacterial concentration of nearly two times higher than that use of atmospheric air in rehydration step did. Effects of oxygen on bacterial survival during rehydration is often underreported [22]. Several factors have been identified, affecting bacterial survival through the rehydration process. These factors include composition [23], volume [24], temperature [22], pH [22] and kinetics of the rehydration media [25]. Rehydration is a final, critical step in recovery of freeze-dried bacteria, enabling reactivation and growth of the cells. Under inappropriate conditions (e.g. presence of oxygen), cells may not be able to repair damages caused by sub-lethal stresses such as oxidative stress. In the present study, rehydration without oxygen protected cells from lethal oxidative stresses.

4. Conclusion

In general, results of the present study have shown that nature of the gases present during the freeze-drying process is a significant factor affecting viability of the two bacteria, *E. coli* and *L. casei*. Atmospheric air during mixing of the bacteria with lyoprotectant and extension during freezing combined with mimetic intestinal gas during rehydration can potentially protect bacteria from oxidative stress. For the starters used in food industries, rehydration with an oxygen-free gas can be easily realized to preserve bacterial viabilities before inoculation into the culture media. For the probiotics used in food industries, it is interesting to realize rehydration in the anaerobic intestinal part of the gut. Such a rehydration can then be controlled using specific encapsulation. Further comprehensive studies are necessary to understand biological mechanisms (e.g. membrane, proteins and antioxidant enzymes) of the gas effects. Since effects of the processing parameters could be species specific, a broader bacterial strain diversity must be studied to complete understanding of this novel concept.

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6. Conflict of interest

No conflict of interest.

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شرایط ویژه گازی برای بهبود قابل ملاحظه زنده مانی *لاکتوباسیلوس کازئی* و *اشرشیا کلی* هنگام خشک کردن انجمادی و بازآبدار کردن

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چکیده

سابقه و هدف: حضور اکسیژن هنگام تولید و بازآبدار شدن[1] آغازگرهای خشک شده به روش انجمادی و زیست‌یارها[2] می‌تواند زنده مانی باکتری ها را کاهش دهد. در واقع، خارج شدن آب از یاخته هنگام خشک کردن انجمادی، می‌تواند اختلال در ساز و کار ضد اکسایشی را افزایش دهد، در نتیجه تنش اکسایشی ناشی از تجمع انواع اکسیژن فعال روی می‌دهد. هدف این مطالعه، نشان دادن چگونگی تاثیر شرایط اتمسفری یا گازی کمتر اکسیداتیو بر زنده مانی دو گونه *لاکتوبا سیلوس کازئی*، باکتری پرکاربرد در زیست فناوری، و *اشرشیا کلی*، باکتری مدل آزمایشگاهی، هنگام خشک کردن انجمادی و بازآبدار کردن بود.

مواد و روش ها: *لاکتوبا سیلوس کازئی* ATCC 334 و *اشرسیا کلی* K12 در محیطی حاوی ۵ درصد جرمی حجمی سوکروز به مدت ۲۴ ساعت به روش خشک کردن انجمادی خشک شدند. دو شرایط گازی (گاز فاقد اکسیژن و هوای اتمسفر) در هنگام مراحل گوناگون فرایند، شامل کشت باکتریایی، اختلاط باکتری با ماده محافظ و بازآبگیری مورد استفاده قرار گرفت. شرایط گازی فاقد اکسیژن با گاز فاقد اکسیژن و حاوی نیتروژن، هیدروژن و دی اکسید کربن ($N_2H_2CO_2$) و محفظه بی هوازی ایجاد شد.

یافته‌ها و نتیجه‌گیری: شرایط گازی تاثیر معنی داری بر میزان زنده مانی باکتریایی داشت ($p< ۰/۰۰۱$ برای *لاکتوباسیلوس کازئی* و *اشرشیا کلی*). جالب اینکه، بر هر دو باکتری، ترکیب بهینه، هوای اتمسفر هنگام اختلاط باکتری با محافظ خشک کردن ($P<۰/۰۰۱$ برای *لاکتوباسیلوس کازئی* و *اشرشیا کلی*) و $N_2H_2CO_2$ هنگام بازآبگیری ($p<۰/۰۰۱$ برای *لاکتوبا سیلوس کازئی* و $P<۰/۰۵$ برای *اشرشیا کلی*) بود. مدیریت شرایط گازی با محافظت باکتری ها از تنش اکسایشی، زنده مانی باکتری ها را هنگام فرایند خشک کردن انجمادی و بازآبگیری (هوای اتمسفری هنگام اختلاط باکتری با محافظ خشک کردن و گاز فاقد اکسیژن هنگام بازآبگیری) افزایش می دهد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- تنش اکسایشی
- گاز فاقد اکسیژن
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[1] rehydration
[2] probiotics