Survival of *Weissella confusa* and *Lactobacillus paracasei* strains in fermented milks under cold storage and after freeze-drying

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The technological properties of two *Lactobacillus paracasei* (UI014 and UI022) and two *Weissella confusa* (UI006 and UI007) strains were studied in order to ascertain their suitability to be used in the formulation of probiotic fermented milks. All strains were able to grow in milk increasing their counts for more than 2 logarithmic units. Additionally, *L. paracasei* strains were able to acidify and coagulate the milk which indicates that they can be employed as starter cultures. However, *W. confusa* strains can only be used as adjunct cultures since their acidification rate was very low. The viability of most strains declined slowly during the cold storage of the dairy product and after 4 weeks, their counts remained approximately 10^7 cfu/ml. Thus, the shelf life of these dairy products is long enough to ensure the intake of viable cells that could promote health benefits. Finally, the four strains studied presented high survival after the freeze-drying process using skim milk as cryo-protectant. Thus this technology can be employed to supply these strains to the dairy industries to be used as starter or adjunct cultures for the formulation of probiotic foods.

Überleben von *Weissella confusa*- und *Lactobacillus paracasei*-Stämmen in fermentierten Milchen unter Kühllagerung und nach der Gefriertrocknung

Die technologischen Eigenschaften von zwei *Lactobacillus paracasei*- (UI014 und UI022) und zwei *Weissella confusa*- (UI006 und UI007) Stämmen wurden mit der Zielsetzung untersucht, ihre Brauchbarkeit für die Herstellung probiotischer fermentierter Milchen zu ermitteln. Alle Stämme waren in der Lage, sich in Milch zu vermehren (Erhöhung der Keimzahlen um mehr als zwei Zehnerpotenzen). Zusätzlich säuerten und koagulierten die *L. paracasei*-Stämme die Milch, was darauf hinweist, dass sie als Säurewecker geeignet sind. Im Gegensatz hierzu können *W. confusa*-Stämme lediglich als Zusatzkulturen eingesetzt werden, da ihre Säuerungsraten sehr niedrig liegen. Die Lebensfähigkeit der meisten Stämme nahm langsam während der Kühllagerung der Produkte ab, und nach vier Wochen blieben die Keimzahlen bei etwa 10^7 KbE/ml. Die Haltbarkeit dieser Milchprodukte ist somit lang genug, um die Aufnahme lebensfähiger Zellen mit möglicherweise gesundheitlichen Vorteilen zu ermöglichen. Die vier untersuchten Stämme zeigten hohe Überlebensraten nach dem Gefriertrocknungsprozess, wobei Magermilch als „Gefrierschutz" diente. Somit kann dieser Technologie zur Versorgung der Milchwirtschaft mit Säurewecker- oder Zusatzkulturen bei der Herstellung probiotischer Lebensmittel empfohlen werden.

62 Fermented milks (probiotic, starter and adjunct cultures)

62 Sauermilchprodukte, probiotisch (Säurewecker und Zusatzkultur)

1. Introduction

The consumption of fermented milk is becoming more popular around the world, although it is a practice that has been in existence from ancient times in different geographical regions. Nowadays, in developed countries there is a remarkable tendency to consume dairy products containing probiotic strains for which beneficial effects have been claimed with different degrees of scientific evidence (1). However, the consumption of probiotic foods in certain developing areas is still scarce and fermented milk market is supported by their nutritional properties more than by other additional health effects. In this sense, in Nigeria the artisanal preparation of fermented milks at home is very frequent; thus, dairy fermentation depends on the activity of bacteria present in the raw milk coming from the environment (2). Currently, in Nigeria the consumption of conventional yogurt are very frequent in urban and even in rural areas. However, the consumption of fermented dairy products due to the health benefits of the bacteria present is not a familiar concept in Nigeria and probiotic foods including strains with proven health benefits are not available in the market. This is confirmed by studies conducted by ANUKAN and co-workers (3, 4) on Nigerian clinicians; 95% of the participants were not familiar with the term probiotics, but 65% indicated their willingness to approve probiotics use for urogenital and gastrointestinal health and a large number of participants preferred probiotic yogurt, suggesting that probiotics would be better accepted if incorporated in food. In fact in developed countries probiotic bacteria (*Lactobacillus* and *Bifidobacterium*) have been incorporated into dairy foods as adjunct cultures and one of the most popular products for the delivery of probiotics is the yogurt (5). Yogurt results from the fermentation of milk with starter cultures of *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus*. A starter can be defined as a "microbial preparation of a large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process" (6). Several lactic acid bacteria (LAB) strains are commonly used as starters because they cause a

rapid acidification of the raw material thus enhancing the microbial safety and shelf life of the fermented product. However, some strains are not able to achieve good acidification rates but they are added as adjunct cultures along the fermentation process since they provide additional sensorial or heath promoting properties.

In previous works carried out by us, two *Lactobacillus paracasei* strains (UI014 and UI022) and two *Weissella confusa* strains (UI006 and UI007) have been isolated from traditional dairy foods from Nigeria and selected because of their antagonistic activities against entero- and uro-pathogens, production of antimicrobial metabolites (organic acids, ethanol, and hydrogen peroxide) and lack of toxic compounds (7, 8). The aim of the current work was to check the technological properties of the aforementioned bacteria through the study of the strain viability in fermented milks under cold conditions and after freeze-drying. This will be the first step to check the suitability of these strains to be produced as starter or adjunct cultures for the formulation of probiotic fermented milks.

2. Materials and methods

2.1 Bacterial strains and culture media

Strains of *L. paracasei* and *W. confusa* were grown in MRS broth (BioKar Diagnostics, Beauvais, France) and were stored in MRS broth containing 20% glycerol at -80ºC. All strains were identified by partially sequencing the 16S rRNA gene (8). As standard procedure, strains from frozen stocks were grown overnight in MRS broth at 37^0C, 5% CO_2 in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) and used to inoculate fresh MRS medium which was incubated for 24 h before use.

2.2 Milk fermentation and strain viability under refrigeration

MRS grown cultures of each strain were washed twice with PBS solution pH 7.0 and used to innoculate (2%) pasteurized skimmed milk (Difco, Becton Dickinson, MD, USA) reconstituted at 11%. Milk fermentation was performed at 37ºC for 24 h in a water bath. Counts of these cultures were determined by making serial dilutions in Ringer (Merck, Darmstadt, Germany) solution and deep-plating on agar-MRS which was incubated for 48 h at 37ºC, 5% CO_2. The results were expressed as colony forming units per milliliter (cfu/ml) of fermented milk. After incubation, the fermented milk was homogenized under sterile conditions, divided into several tubes and stored at 4ºC for 28 days. Counts of viable cells after 7, 14, 21 and 28 days were carried out as previously indicated. The pH after 24 h of milk fermentation and after 7, 14, 21 and 28 days of cold storage was directly measured with pHmeter Symphony VWR SB70P (ThermoFisher, USA).

2.3 Effect of freeze-drying on strain viability

Cells from MRS cultures grown for 24 h were harvested by centrifugation (2,800 *g*, 4°C, 20 min), washed once with PBS buffer and concentrated 10 times in 1 ml of pasteurized skimmed milk. Cells suspended in milk were kept at -80ºC in sterilized glass tubes for 24 h and afterwards they were lyophilized in a Freezemobile 12EL equipment (VirTis, Gardiner, NY, USA) for 24 h. Finally, the lyophilized cells were dissolved in saline solution in the same initial volume (1 ml). The number of viable cells before and after lyophilization was determined by counting in agar-MRS as previously described. Results were expressed in cfu/ml and this data were used to calculate the percentage of survival.

2.4 Statistical analysis

Data of survival were analyzed by means of several one-way ANOVA tests using the SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA). The ANOVA factor used was the strain type with four categories: UI006, UI07, UI014 and UI022. Afterwards, the differences among the four strains were assessed by the mean comparison analysis LSD (least-significant difference, $p < 0.05$).

3. Results and discussion

3.1 Growth of the LAB in milk

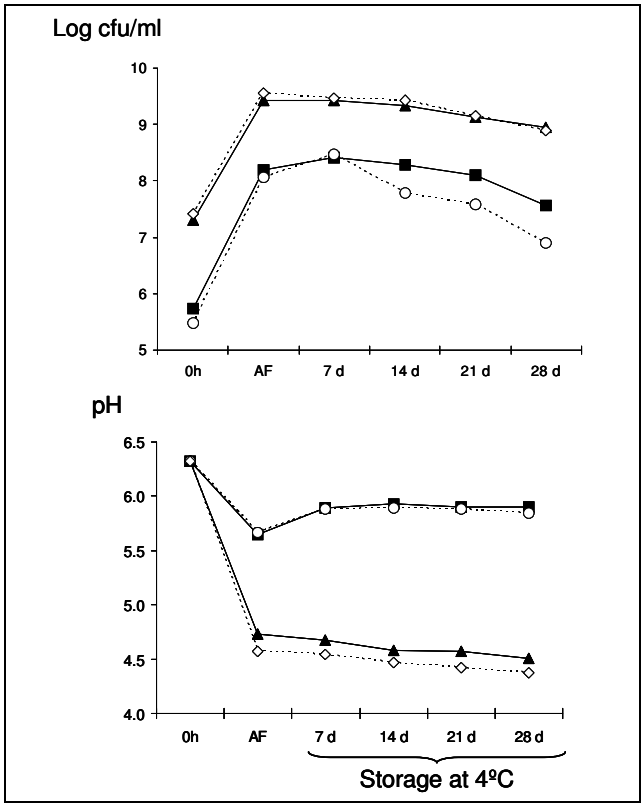

Fig. 1: Growth (Log cfu/ml) of *W. confusa* and *L. paracasei* strains in milk and pH values of milks after fermentation period (AF = 24 h of incubation at 37ºC) and along the storage of fermented milks at 4ºC for 28 d. (◊) *L. paracasei* UI022, (▲) *L. paracasei* UI014, (■) *W. confusa* UI006 and (○) *W. confusa* UI017.

The capability of the strains under study to grow in milk was determined in order to assess their suitability for use as starters or adjunct cultures in the formulation of fermented dairy foods for probiotic use. The number of viable cells produced by each LAB in milk after fermentation at 37ºC for 24 h is presented in Fig. 1. All strains were able to increase their counts for more than 2 log units: 2.44±0.07 and 2.52±0.05 for *W. confusa* UI006 and UI007, and 2.12±0.06 and 2.14±0.07 for *L. p*aracasei UI14 and UI22, respectively. This indicates a similar capability of the strains

Table 1: Percentage of survival (refer to the cfu/ml after fermentation) of *W. confusa* and *L. paracasei* strains in fermented milks stored at 4ºC for 28 days. Differences among strains in each day of cold storage have been assessed by means of one-way ANOVA. The strains that do not share a common superscript within the same day are statistically different (p < 0.05) accordingly to the LSD (least-significant difference) mean comparison test (Mean ± SD)

Species	Strain	7 days	14 days	21 days	28 days
W. confusa	UI006	170.9±29.0^{b}	122.9±16.5^{c}	82.9±2.3^{d}	24.5±5.2^{b}
	UI007	260.9±17.4^{c}	53.3±9.8^{a}	33.0±4.3^{a}	6.9±0.0^{a}
L. paracasei	UI014	101.9±18.9^{a}	82.1±4.7^{b}	50.4±0.6^{c}	33.9±0.8^{c}
	UI022	83.3±11.1^{a}***	76.1±18.6^{ab}**	38.8±1.0^{b}***	21.8±3.2^{b}***
One-way ANOVA: ** p < 0.01, *** p <0.001					

of both genera to growth in milk which is in accordance with other studies reported in literature for probiotic strains (9). However, the pH values produced in milk fermented with *W. confusa* strains were significantly higher (p<0.001) than that of *L. paracasei* strains which reflects differences in their metabolic activities in milk (Fig. 1). It seems that *L. paracasei* strains are producing higher amounts of organic acids, which correlates with a lower pH in the fermented milks, than *W. confusa* strains. In this way, it is known that *L. paracasei* displays a homofermentative metabolism of glucose, thus producing lactic acid as main end product. Whereas, *W. confusa* is heterofermentative and thereby other metabolites in addition to organic acids can be formed (10). In fact, we have recently shown that the main end product of glucose metabolism of our *Weissella* strains growing in MRS broth was ethanol and lactic acid while that of *L. paracasei* was lactic acid (8). Thus, similar behaviour could be expected in milk medium. The *W. confusa* strains were able to grow in milk to 10^8 cfu/ml, while the *L. paracasei* strains reached 10^9 cfu/ml. The suggested daily intake for probiotic strains is around 10^9 viable cells per day (11), which could correspond to around 10^7 to 10^8 cfu/ml or g of product. Therefore, the LAB used in this study can reach enough number of viable cells and could be used to formulate probiotic fermented milk, if some health benefits could be attributed to them. In addition, since the *L. paracasei* strains can acidify the fermented milk to pH below 5.0 (4.73±0.03 and 4.57±0.14 for U0I14 and UI022, respectively) these strains can be use as starters for coagulation of milk. But, *W. confusa* strains were not able to form a hard milk-coagulum because they were not able to acidify the milk below the precipitation point of the casein (5.65±0.01 and 5.67±0.01 for UI006 and UI007, respectively). Thus *Weissella* strains can be used as adjunct cultures to be added in combination with, for example, yoghurt starter cultures.

3.2 Effect of storage at 4ºC on viability of LAB in fermented milk

Probiotic capability is a strain dependent characteristic and it is well accepted that in order to confer health benefits, a given probiotic strain should be present in the medium in enough amounts and in a viable state (12). The survival of probiotics in functional dairy products has improved in recent years but the loss of bacterial viability increases with the storage period of the commercial products (13). In the current work, we have studied the survival of the four strains in fermented milks during the cold storage in order to ascertain the shelf life of the product. The growth of the LAB in fermented milk as well as the evolution of their pH values during cold storage at 4ºC is depicted in Figure 1. In general, the pH values of the fermented milk remained stable during the storage period, indicating absence of excessive acidification in the case of *L. paracasei* strains (28 days pH values 4.51±0.01 and 4.37±0.11 for UI014 and UI022, respectively). A slight increase of pH was detected in milk fermented with *W. confusa* strains after 7 days of cold storage, which was also coincidental with a slight increase of the bacterial counts for both UI006 and UI007 strains (Log cfu/ml increase 0.23±0.01 and 0.45±0.03, respectively). This increase in pH was probably due to the production of metabolites coming from the milk protein breakdown since the lysis of the bacteria could be discarded given the increase in the counts detected. Afterwards, the pH of milk fermented with *Weissella* strains was stable without changes (28 days pH values 5.91±0.01 and 5.85±0.01 for UI006 and UI007, respectively). Regarding the viability of the strains along the cold storage period, the percentage of survival was calculated with respect to the counts obtained after the fermentation (Table 1). Statistical differences were detected among strains in each storage period tested ($p \leq 0.01$) thus indicating that the loss in viability was strain dependent (13). After 7 days of storage, *Weissella* strains kept their viability, even some strains were able to slightly grow. However, strain *W. confusa* UI007 was quickly loosing its viability; after 14 days of cold storage it was reduced to half of the initial values and a considerable decrease was obtained at the end of the cold storage ($8.0x10^6$ cfu/ml). On the contrary, *W. confusa* UI006 and the two *L. paracasei* UI014 and UI022 strains were loosing their viability slowly and at the end of storage (28 days) the counts of each strain were $3.8x10^7$, $9.0x10^8$ and $7.9x10^8$ cfu/ml, respectively. Thus, most strains were still viable in the desired amount in the fermented milk after 4 weeks of cold storage which is a desirable property if these strains are intended for oral delivery probiotic use. The first articles reporting the viability of probiotic strains in dairy fermented products showed a constant decline in the bacterial counts under cold storage (14). However, the improved technologies as well as the selection of strains with higher tolerance levels of stressing technological factors (15) increased the self life period of the dairy probiotic products (13). Thus the selection of strains based on their ability to grow in milk and to survive the storage period of the food are important criteria for the selection of strains in combination with their probiotic properties.

3.3 Effect of lyophilization on the viability of LAB

In order to use LAB strains for food probiotic applications, either as starter or adjunct cultures, some preservation methods are necessary to supply the bacteria to the industries. Frozen direct-to-vat cultures require very low temperatures for transportation and storage which limits their application to some developed areas or countries. Thereby, freeze-drying is a technology more suitable because it does not require freezing conditions for distribution (16). Therefore, the effect of the freeze-drying process on the viability of our potential probiotic strains was tested. Statistical differences ($p<0.001$) on viability after lyophilization were detected among strains (Fig. 2).

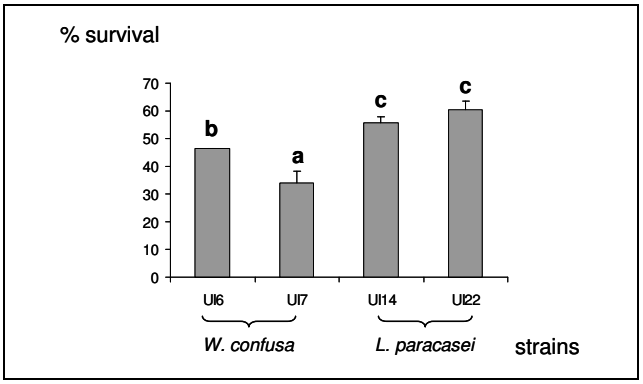

Fig. 2: Percentage of survival of *W. confusa* and *L. paracasei* strains, suspended in skimmed milk as cryoprotective, after lyophilization. Differences among strains have been assessed by means of one-way ANOVA. The strains that do not share a common superscript are statistically different ($p < 0.05$) accordingly to the LSD (least-significant difference) mean comparison test.

L. paracasei strains presented the highest survival rates whereas *W. confusa* strains showed the lowest, UI007 being the strain with the highest viability reduction (66%). However, these survival percentages represent a reduction lower than 0.5 logarithmic units (Log cfu/ml reduction of 0.33±0.01 and 0.47±0.05 for *W. confusa* UI006 and UI007, and 0.25±0.02 and 0.22±0.02 for *L. paracasei* UI014 and UI022, respectively) which indicates that all bacteria tested survived the freeze-drying process well. The lyophilization is a process that involves two steps, first a frozen period followed by a dry step by sublimation under high vacuum. Thus, bacteria are submitted to stressing conditions and the response to this challenge depends on the strain (17). In this way, the use of different cryoprotectants, such as skim milk in our case, could help to improve the survival rates of bacteria (18).

4. Conclusion

This study was conducted to determine if four LAB selected according to their probiotic potential have suitable technological properties to be included in fermented milk products. All strains were able to grow in milk but only *L. paracasei* strains acidified the milk in order to produce a fermented product. Thus, these strains could be used either as starters or as adjunct cultures, whereas *W. confusa* strains could be added as adjunct cultures in combination with other starters for the manufacture of fermented milk such as yoghurt. After 4 weeks of cold storage, the counts of viable cells of most strains in the fermented milk was 10^7 cfu/ml. This is the minimum amount of daily intake recommended by some authors to obtain health benefits. Thus, the shelf life of milk containing these bacteria is high which makes the application of the strains studied suitable for oral delivery as putative probiotic foods. Finally, all bacteria tested showed high percentages of survival after freeze-drying. Thereby, this process can be applied to obtain enough cell biomass that will provide starter or adjunct cultures for the dairy industries.

Acknowledgements

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