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# Quantitative analysis and molecular typing with pulsed-field gel electrophoresis of two probiotic *Lactobacillus* strains from sour milk products available on the Swiss food market

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## Introduction

At the end of the eighties, the first probiotic foods appeared on the market in Switzerland. These pioneer products were yoghourts and sour milks supplemented with bacteria of the genus *Bifidobacteria*. In the following years, products with probiotic strains of lactobacilli became available to the consumers. Although probiotic foods are now well established since more than a decade, their legal regulation is still rudimental. The Swiss Food Legislation only decrees that probiotic microorganisms in milk products have to be present in a number of at least $10^6$ living cells per gram (1). This minimal requirement was set up to protect the consumers from misleading. Furthermore, there should be no discrepancy between the microorganisms named on the labels and those which are added to or grown in the products. To examine these aspects, both methods for quantitative analysis and for differentiation of bacterial strains are needed. In Switzerland, conformity of foods with legal microbiological criteria is examined with official methods published in the Swiss Food Manual (2). However, for the microbiological analysis of probiotic foods, official methods are not established yet. It was the purpose of the present study to evaluate analytical tools for the quantitative analysis and molecular identification of probiotic lactobacilli used in foods. Based on the obtained results, elements for a comprehensive legal regulation of probiotic foods, including microbiological quality aspects, are suggested.

## Materials and methods

### *Bacterial strains*

53 strains of *Lactobacillus rhamnosus* were isolated and described in a previous study (3). Reference strain *Lactobacillus johnsonii* NCC 533 (LC1) was made available by Nestec Ltd., Vevey, Switzerland, and *Lactobacillus rhamnosus* GG (LGG) by Valio Ltd., Valio, Finland.

### *Quantitative analysis of two Lactobacillus strains from sour milk on MRS agar*

13 samples of sour milk containing probiotic lactobacilli of two producers were purchased in local super markets and kept at 4 °C until examined. The products were advertised to contain either LC1 or LGG. At the expiry date, 10 g of sample and 90 ml of 0,9 % NaCl-solution were homogenized in a stomacher, serially diluted to $10^{-7}$ and quantitatively analyzed as follows: 1 ml of each dilution was given into petridishes, liquid MRS agar (Oxoid CM 361) added and the poured plates incubated. After 48 h at 37 °C under anaerobic conditions (AnaeroGen, Oxoid AN35), target colonies were counted and the number per gram of product calculated. Colonies of LGG and LC1 are supposed to be white with a diameter of 2–3 mm. From each sample, a colony of the target organism was subcultered twice on MRS agar and frozen at –70 °C until further used.

### *DNA preparation from lactobacilli for strain-typing by pulsed-field gel electrophoresis (PFGE)*

Strains to be typed were prepared by regrowing –70 °C – cultures in MRS broth (Oxoid CM 359) for 48 h at 37 °C followed by a culture on MRS agar for 48 h at 37 °C under anaerobic conditions. Bacterial cells were harvested with an inoculation loop, homogenously suspended in 1 ml of TE-buffer (10 mmol/l Tris/100 mmol/l EDTA/pH 8.0) and the $OD_{600}$ measured with a 1:100 dilution in 0.9 % NaCl-solution. Based on the obtained value, the volume of TE-buffer was calculated which has to be added to the original cell suspension in order to reach an $OD_{600}$ of 2.5.

0.3 ml of 1.5 % agarose (BMA 1-800-341-1574) in TE-buffer, adjusted to a temperature of 60 °C, was mixed with 0.3 ml cell suspension and immediately poured into a plug mold of 1×5×8 mm. Solidified agar plugs were transferred into Eppendorf reaction tubes containing 1.5 ml TE-buffer with 25 mg/ml of lysozyme (Sigma L6876) and 20 U/ml of mutanolysin (Sigma 9901) and rotated overnight at 10 rpm (Hybaid Mini 10, MGW-Biotech) at 37 °C. After washing the plugs twice in TE-buffer for 15 min, 1.5 ml of 0.5 mmol/ml EDTA, 0.01 mg/ml N-Laurolylsarcosine (pH 8) and 2 mg/ml proteinase K (Boehringer 745723) was added and incubated overnight at 50 °C under rotation. Subsequently, the plugs were washed five times in 5 ml TE-buffer for one hour under rotation, cut and one half equilibrated in 0.3 ml restriction-buffer (delivered together with restriction enzymes), transferred into

0.2 ml restriction buffer with 40 U of the specific enzyme, and incubated for 5 h at 37 °C. DNA from strains of LGG was digested with *Not* I (Roche 1014714) and DNA from LC1 with *Sma* I (Roche 220566).

For the electrophoresis (CHEF-DR III device, Bio-Rad) of DNA from *L. rhamnosus*, the following protocol of *Tynkkynen et al.* (4) was applied: gel: 1 % agarose in 0.5 × TBE equilibrated for 30 min at 14 °C before electrophoresis; initial switch time: 1 s; final switch time: 15 s; run time: 22 h; field: 5 V/cm; angle: 120 °.

For the electrophoresis of DNA from LC1 the following parameters were applied: gel: 1.1 % agarose in 0.5 × TBE equilibrated for 30 min at 14 °C before electrophoresis; initial switch time: 2 s; final switch time: 30 s; run time: 24 h; field: 5,3 V/cm, angle: 120 °.

On every gel, a DNA-standard in the size range of 0.1 to 200 kb was run (Biolabs N0350S). After electrophoresis, gels were stained in ethidiumbromide (1 µg/ml TBE) for 30 min, rinsed twice in distilled water, the restriction patterns visualized under UV light (302 nm) and documented with a digital camera (Gel Print 2000i, MWG-Biotech).

### *Test system for the discriminatory power and reproducibility of PFGE*

To estimate the discriminatory power of the applied typing method, 53 epidemiologically unrelated strains of *L. rhamnosus* from a previous study were analyzed (3). The resulting restriction patterns were compared by eye. Those strains showing highly similar patterns were reanalyzed together on separate gels. The discriminatory power was calculated according to a procedure described by *Hunter and Gaston* (5) which is based on Simpson's index of diversity, a method developed for the description of species diversity within an ecological habitat. Reproducibility was tested by analyzing strain LGG 8-times over the period of one year. For each analysis, the strain to be tested was regrown from a stock culture kept at –70 °C.

## Results and discussion

### *Discriminatory power and reproducibility of PFGE*

Typing systems should be easy to perform, have a high discriminatory power, and give reproducible results. Unfortunately, we did not succeed to find a method which fulfills these three criteria perfectly well. PFGE is rather labourcosty but in comparison with ribotyping and random amplified polymorphic DNA (RAPD), it was shown to have the highest discriminatory power (4). We have given preference to this aspect and therefore selected PFGE for typing experiments. To test the method's performance, we analyzed 53 epidemiologically unrelated strains of *L. rhamnosus* isolated and described in a previous work (3). Among the 53 strains, 43 different patterns could be differentiated which represented a discriminatory index of 0.988 according the procedure of *Hunter and Gaston* (5). Such high discriminatory power could only be reached by combined methods as for example

bacteriocin- and phage typing (6). In a recent study, *Wei and Chiou* typed *Staphylococcus aureus* strains from food borne outbreaks with PFGE and three PCR-typing techniques and clearly showed PFGE to be the most discriminatory method (7).

Figure 1 shows a selection of ten *L. rhamnosus* strains with different PFGE-patterns. Sources of the tested isolates were cheese, raw milk and a probiotic product. One strain originated from an official culture collection. From the strain belonging to lane 8 (LGG), typing was done 8-times over the period of one year (data not shown). These analyses entirely revealed identical patterns as in lane 8 and thus showed a solid reproducibility for the applied PFGE-protocol.

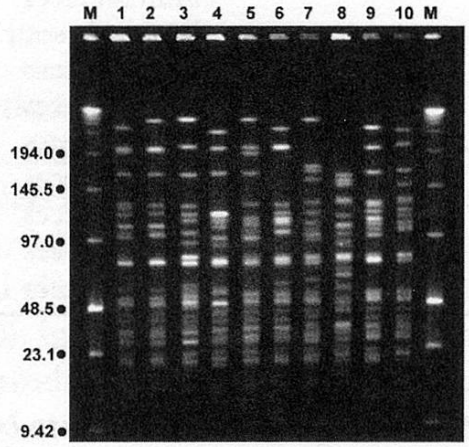



Figure 1 **Heterogeneity of *Lactobacillus rhamnosus* strains of different origin shown by PFGE-profiling (*Not* I digest)**
(Lanes 1, 2, 6 and 7: isolates from cheese; lanes 3, 4 and 5: isolates from raw milk; lane 8: strain LGG; lane 9: clinical isolate; lane 10: strain NCTC 10302; lanes M: molecular size marker, range 0.1 to 200 kb).

### *Relation of L. rhamnosus strains of different origin*

Seven restriction patterns could be demonstrated with more than one strain. Table 1 shows that common patterns were mostly found with strains from human feces. Three times however (profiles A, C and D), identical strains were found in strains both from feces and foods. In one of these cases, a strain from feces was indistinguishable from LGG isolated from a probiotic milk product. The fecal sample where LGG originated from was anonymized and it was therefore not possible to find out whether the person belonging to the sample consumed probiotic products containing the respective microorganism. Nevertheless, the obtained result showed PFGE to be a suitable analytical tool to trace back probiotic strains even to the intestinal bacterial flora. *Rautio et al.* successfully applied PFGE (*Not* I and *Sfi* I digests) to demonstrate identity between LGG and a *Lactobacillus* strain isolated from a liver abscess of a 74-year-old lady. This report showed LGG for the first time as the causative agent of a sporadic case of human infection (8).

Table 1
**Typing of 53 isolates of *Lactobacillus rhamnosus* with PFGE and origin of strains with identical profiles**

| *Identical PFGE-profile* | *Origin of strains* |
|---|---|
| A | Semi-hard cheese |
| | Human feces |
| | Human feces |
| | Human feces |
| | Human feces |
| B | Gruyère cheese |
| | Gruyère cheese |
| C | Raw milk |
| | Human feces |
| D | Probiotic sour milk |
| | Human feces |
| E | Human feces |
| | Human feces |
| F | Human feces |
| | Human feces |
| G | Human feces |
| | Human feces |

### *Counting and analysis of presumed Lactobacillus colonies from MRS agar*

At present, the market for foods with probiotic lactobacilli in Switzerland is dominated by sour milks supplemented with either LGG or LC1. In total, we quantitatively analyzed 13 samples with these microorganisms at the expiry dates. In products with *L. johnsonii*, there were no other lactobacilli, and cocci (streptococci or lactococci) appeared only in form of pin point colonies on MRS-agar. Some products with LGG also contained *Lactobacillus acidophilus*. However, the two species could be differentiated unquestionably because of their particular colony morphology since colonies of *L. rhamnosus* were white with a diameter of 2–3 mm and colonies of *L. acidophilus* were grey-white with a diameter of around 1 mm. Table 2 shows that all the samples fulfilled the minimal requirement of $10^6$ living cells per gram at the expiry date and were therefore conform with the law (1). There are two explanations for the high performance of the examined foods. Firstly, the analyzed sour milks were all produced by industries known to have solid systems of quality control. Secondly, in contrast to bifidobacteria, lactobacilli do not rapidly die off in sour milk products (9). After quantitative bacteriological analysis, confirmation tests are usually done in order to get identification on the species level. This step could be omitted because presumptive colonies and reference strains were subsequently typed with PFGE.

Table 2
**Counts of *Lactobacillus johnsonii* (LC1) and *Lactobacillus rhamnosus* GG (LGG) in different probiotic sour milks at the end of expiry dates**

| *Products with LC1* | *cfu/g* | *Products with LGG* | *cfu/g* |
|---|---|---|---|
| 1 | $7.2\times10^7$ | 1 | $5.4\times10^7$ |
| 2 | $2.7\times10^7$ | 2 | $5.5\times10^7$ |
| 3 | $6.6\times10^7$ | 3 | $3.0\times10^7$ |
| 4 | $8.2\times10^7$ | 4 | $3.0\times10^7$ |
| 5 | $8.0\times10^7$ | 5 | $7.0\times10^7$ |
| 6 | $4.0\times10^7$ | 6 | $3.9\times10^7$ |
| 7 | $4.5\times10^7$ | | |

cfu: colony forming unit

### *Identification of probiotic lactobacilli from foods*

In probiotic products, those strains mentioned on the labels have to be present in the products and vice versa. Unfortunately, this quality aspect is not always fulfilled. *Hamilton-Miller et al.* analyzed eleven so called probiotic bio-yoghourts from the UK-market and showed that none of them gave information about the amounts of organisms (10). In the present work, no discrepancies between the indications on the labels and product contents could be found. We isolated the probiotic strains from 13 samples of sour milk and compared them by PFGE-fingerprinting with the reference strains obtained by the producer companies. In each case, the target organism could be demonstrated in the products as illustrated with two examples in figures 2 and 3.

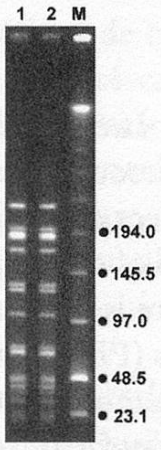
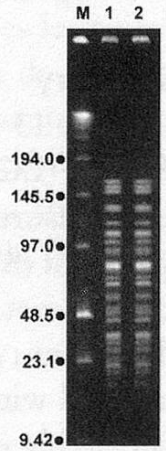



Figure 2 ← **Identification with PFGE (*Sma* I digest) of *Lactobacillus johnsonii* (LC1) from a probiotic sour milk by comparison with a reference strain**

Figure 3 → **Identification with PFGE (*Not* I digest) of *Lactobacillus rhamnosus* GG (LGG) from a probiotic sour milk by comparison with a reference strain**
(Lane 1: product strain; lane 2: reference strain; lane M: molecular size marker, range 0.1 to 200 kb)



## Conclusions and reflections on the official control of probiotic microorganisms

To assure the quality of probiotic foods, specific legal regulations are needed. Combined with appropriate analytical techniques, such regulations would lead to full transparency and improve consumer protection with regard to health risks and misleading. Regulations should include a definition of the term "probiotic microorganism", lay down minimal amounts for the active organisms per gram of product and define what kind of scientific data are needed to proof appraisals. Regulations should also clearly require that added microorganisms must be safe for human consumption and be free of transmissible resistances to antibiotics; a requirement which is mandatory in case of probiotic microorganisms in animal feeds (11). To protect consumers from health risks and misleading, it would be indicated that probiotic products, prior to enter the market, are approved by the competent authorities and that the above mentioned aspects are carefully examined in this context. Producer companies should demonstrate that quantitative minimal requirements for the active microorganisms are met by their products and that the origin of the added strains is ascertained. For this purpose, adequate methods for quantitative analysis and strain typing should be available and be presented to the competent authorities on demand together with reference strains of the probiotic microorganisms. The competent authorities should verify the data supplied by the producers with laboratory testing within the authorization procedure, and later by random testing of market samples. For species identifications, for comparisons of strains and to trace back probiotic bacteria, PFGE-typing seems to be a suitable method because it is highly discriminating and reproducible. However, future work is needed to simplify this technique and to make it applicable for the whole range of probiotic bacteria on the market. Preferably, such work should be done within the framework of an international organization such as ISO.

## Summary

From 13 sour milks of two producers supplemented with either *Lactobacillus johnsonii* (LC1) or *Lactobacillus rhamnosus* (LGG), counts of active microorganisms were measured on MRS agar. In each case, the legal minimal requirement of $10^6$ living cells of the probiotic microorganism per gram of product at the end of the expiry date was met. From all the analyzed samples, the probiotic strains were isolated for subsequent typing. As typing method, pulsed-field gel electrophoresis (PFGE) was evaluated with 53 epidemiologically non-related strains of *L. rhamnosus* and found to be highly discriminating and reproducible. All LC1 or LGG strains isolated from products could be shown to have the same PFGE-profile as the corresponding reference strains. These results identified PFGE as a suitable tool to identify probiotic lactobacilli in the context of official food control and for epidemiological trace back. Finally, considerations with regard to the legal regulation of probiotic products, including analytical procedures for strain identification, were made.

## Zusammenfassung

Von 13 probiotischen Sauermilcherzeugnissen zweier Hersteller mit *Lactobacillus johnsonii* (LC1) oder *Lactobacillus rhamnosus* (LGG) wurde die Gesamtzahl aktiver Keime auf MRS Agar ermittelt. In allen Fällen lagen die Ergebnisse am Ende der Verbrauchsfrist über der amtlich festgelegten Minimalanforderung von $10^6$ lebenden Zellen pro Gramm Produkt. Aus jeder Probe wurde der probiotisch aktive Stamm zur nachfolgenden Typisierung isoliert. Als Typisierungsverfahren wurde Pulsed-Field Gel-Elektrophorese (PFGE) mit 53 epidemiologisch nicht verwandten Stämmen von *L. rhamnosus* validiert und dabei eine solide Reproduzierbarkeit der Ergebnisse und eine hohe Aufschlusskraft ermittelt. Sämtliche aus den Produkten isolierten Stämme von LC1 und LGG ergaben PFGE-Profile, die mit denjenigen der entsprechenden Referenzstämme übereinstimmten. Die erhaltenen Ergebnisse identifizierten PFGE als geeignetes Verfahren zur Überprüfung probiotischer Keime im Kontext amtlicher Kontrollen und als Instrument zur epidemiologischen Rückverfolgbarkeit. Abschliessend werden allgemeine Überlegungen für eine verbesserte gesetzliche Regelung probiotischer Erzeugnisse gemacht und in diesem Kontext der Einbezug von Verfahren zur Stammidentifizierung gefordert.

## Résumé

13 laits acidulés probiotiques ont été analysés sur MRS agar quant à leur teneur en *Lactobacillus johnsonii* (LC1) ou *Lactobacillus rhamnosus* (LGG). Pour tous les échantillons, la teneur minimale requise par la législation, soit $10^6$ cellules vivantes par gramme de produit, était dépassée à la date limite de vente. Les souches probiotiques ont été isolées à partir de chaque échantillon en vue d'une typisation ultérieure. Dans ce but, l'électrophorèse en champ pulsé (PFGE) a été choisie et validée à l'aide de 53 souches *Lactobacillus rhamnosus* isolées ultérieurement dans le cadre d'une étude épidémiologique. Un haut pouvoir discriminant et une solide répétabilité font de cette méthode un outil de choix pour la typisation. Toutes les souches isolées présentent un profil identique à ceux obtenus avec les souches de référence. Les résultats montrent que le PFGE est une méthode très performante, et pour l'identification des souches probiotiques et comme instrument de traçabilité épidémiologique. Enfin, une réflexion générale est donnée sur la nécessité d'améliorer la réglementation des produits probiotiques et d'y inclure une méthode permettant l'identification des souches.

## Acknowledgements

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**Key words**

*Lactobacillus rhamnosus, Lactobacillus johnsonii,* probiotic foods, PFGE, legislation

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