Democritus University of Thrace
Department of Molecular Biology and Genetics

Μοριακή Βιοτεχνολογία και Διατροφή

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Μοριακές τεχνικές με εφαρμογή στη Βιοτεχνολογία τροφίμων

Σε αντίθεση με τις προϋπάρχουσες κλασικές μικροβιολογικές τεχνικές που βασίζονται κυρίως στη φαινοτυπική καταγραφή και παρατήρηση, με αποτέλεσμα τη περιορισμένη διακριτική ικανότητα, οι μοριακές τεχνικές στηρίζονται στη γονοτυπική ανάλυση των μικροοργανισμών. Πιο συγκεκριμένα, πραγματοποιούνται μελέτη και χαρακτηρισμός των γενετικών δεικτών των μικροοργανισμών μέσω κατάλληλης επεξεργασίας του γενετικού τους υλικού (DNA). Μια από τις πιο αποτελεσματικές και αξιόπιστες τεχνικές είναι η δοκιμή της Πολλαπλής Αλυσιδωτής Αντίδραση Πολυμεράσης (Multiplex Polymerase Chain Reaction – MPCR).

Η δοκιμή της Πολλαπλής Αλυσιδωτής Αντίδραση Πολυμεράσης (Multiplex Polymerase Chain Reaction – MPCR)

Αρχικά, γίνεται συγκριτική μελέτη της αλληλουχίας ενός καθολικού γονιδίου με σκοπό την ανίχνευση πολυμορφικών θέσεων, δηλαδή χαρακτηριστικών για το στέλεχος προς διερεύνηση γενετικών περιοχών. Στη συνέχεια, βάση των πολυμορφικών αυτών θέσεων σχεδιάζονται οι αντίστοιχοι εκκινητές για τη δοκιμή MPCR. Παράλληλα γίνεται απομόνωση γενετικού υλικού από τους μικροοργανισμούς, το οποίο θα χρησιμοποιηθεί ως μήτρα για την αντίδραση πολυμερισμού. Η αντίδραση πραγματοποιείται σε ειδική συσκευή (θερμικός κυκλοποιητής) και το αποτέλεσμα μπορεί να γίνει γνωστό σε λιγότερο από μία ώρα. Η MPCR χαρακτηρίζεται για τη ταχύτητα, την υψηλή επαναληψιμότητα και τη διακριτική ικανότητα της. Πρόσφατα, έχει χρησιμοποιηθεί για την ανίχνευση του *L. casei* ATCC 393 σε καλλιέργειες προβιοτικών τροφίμων αλλά και σε βιολογικά δείγματα.

Multiplex PCR: Critical Parameters and Step-by-Step Protocol

BioTechniques 23:504-511 (September 1997)

O. Henegariu, N.A. Heerema, S.R. Dlouhy, G.H. Vance and P.H. Vogt[1]
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Optimization of Multiplex Reaction Components

Amount of primer (Step 5, B and C). Initially, equimolar primer concentrations of 0.2–0.4 μM each were used in the multiplex PCR (Figure 3c), but there was uneven amplification, with some of the products barely visible even after the reaction was optimized for the cycling conditions. Overcoming this problem required changing the proportions of various primers in the reaction, with an increase in the amount of primers for the "weak" loci and a decrease in the amount for the "strong" loci. The final concentration of the primers (0.04–0.6 μM) varied considerably among the loci and was established empirically.

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dNTP and $MgCl_2$ concentrations (Step 5D).

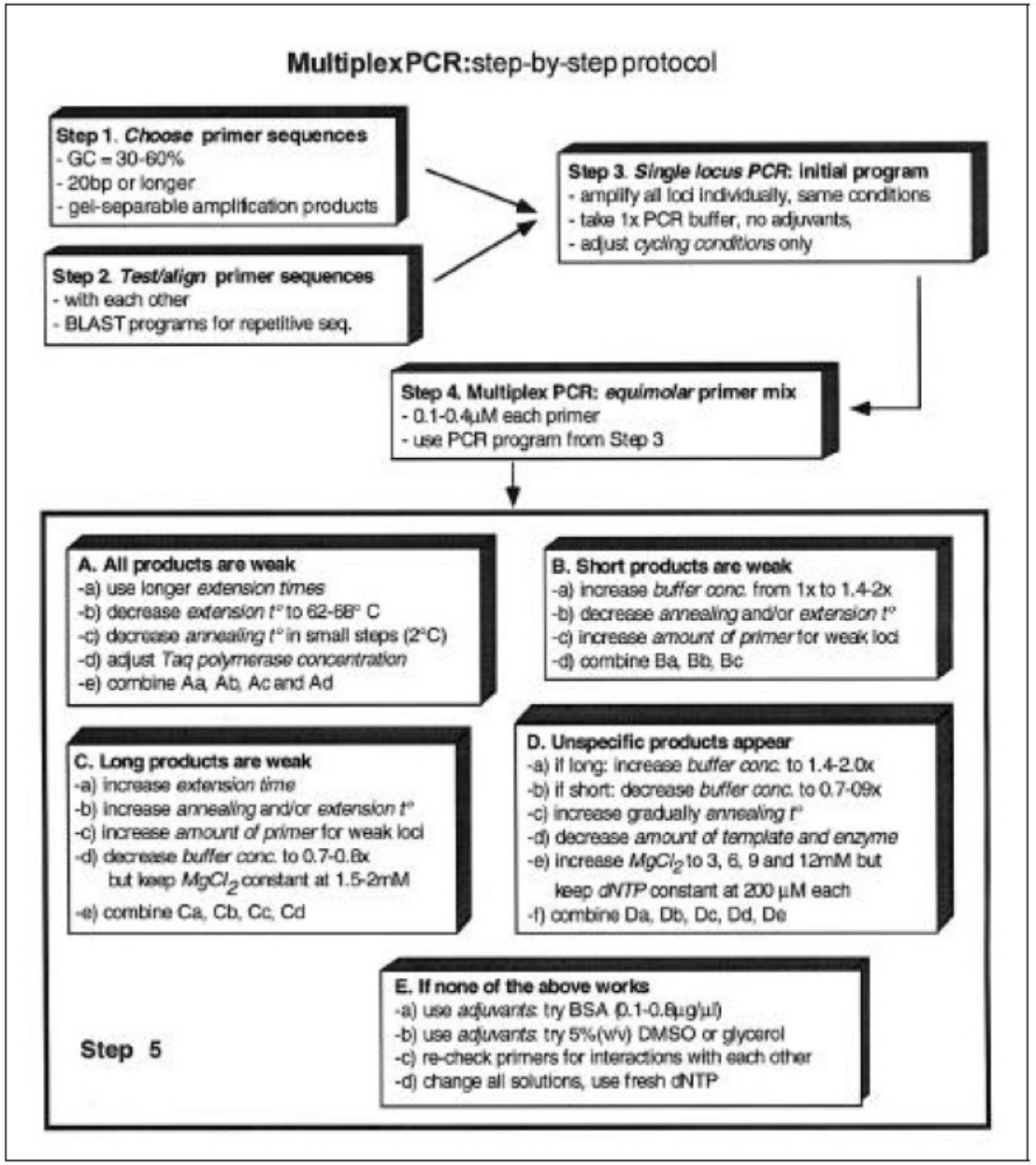
dNTP. The significance of the dNTP concentration was tested in a multiplex PCR test with primer mixture Y-4. Magnesium chloride concentration was kept constant (3 mM), while the dNTP concentration was increased stepwise from 50–1200 μM each (Figure 4b). The best results were at 200 and 400 μM each dNTP, values above which the amplification was rapidly inhibited. Lower dNTP concentration (50 μM) allowed PCR amplification but with visibly lower amounts of products. dNTP stocks are sensitive to thawing/freezing cycles. After 3–5 such cycles, multiplex PCRs often did not work well; products became almost completely invisible. To avoid such problems, small aliquots (2–4 μL, 10–20 reactions) of dNTP (25 mM each) can be made and kept frozen at -20°C and centrifuged before use. This "low stability" of dNTP is not so obvious when single loci are amplified.

$MgCl_2$. A recommended magnesium chloride concentration in a standard PCR is 1.5 mM at dNTP concentrations of around 200 μM each. To test the influence of magnesium chloride, a multiplex PCR (mixture Y-3) was performed, keeping dNTP concentration at 200 μM and gradually increasing magnesium chloride from 1.8–10.8 mM (Figure 4c). Amplification became more specific (unspecific bands disappeared), and the products acquired comparable intensities (at 10.8 mM). In PCRs with up to 20 mM $MgCl_2$, products became barely visible, as if the reactions were inhibited (not shown).

Amount of template DNA and *Taq* DNA polymerase (Step 5, A and D). At DNA template quantities between 30 and 500 ng/25 μL reaction, mixture Y-3* showed no significant differences (Figure 4f); however, below 30 ng the amount of some of the products decreased. When the amount of template DNA is very low (pg of DNA), efficient and specific amplification can be obtained by further lowering the annealing temperature, sometimes by as much as 10°–12°C (data not shown).

Different concentrations of *Taq* DNA Polymerase (Perkin-Elmer) were tested using primer mixture Y-3 (Figure 5a). The most efficient enzyme concentration seemed to be around 0.4 μL or 2 U/25 μL reaction volume. Too much enzyme, possibly because of the high glycerol concentration in the stock solution, resulted in an unbalanced amplification of various loci and a slight increase in the background. Five native *Taq* DNA polymerases, from five different sources, performed similarly on mixture Y-4 in 1.6× PCR buffer using 2 U/25 μL (Figure 5b).

We have presented a series of examples of testing various parameters to optimize multiplex PCR. Optimal combination of two of these parameters, annealing temperature and KCl (salt) concentration, is essential in any PCR to obtain highly specific amplification products. Magnesium chloride concentration needs only to be proportional to the amount of dNTP, and these values can be constant for any reaction. Although gradually increasing magnesium chloride concentrations may further influence the reaction, the other two parameters mentioned seem to be much more important in obtaining specific, high yields of PCR product(s). In multiplex PCR, adjusting primer amount for each locus is also essential. Figure 1 illustrates a rational approach for developing efficient multiplex PCRs. The list of various factors that can influence the reaction is by no means complete. Nevertheless, optimization of the parameters as presented in this work should provide a basic way of approaching some of the common problems of multiplex PCR.


Multiplex PCR: step-by-step protocol
Step 1. Choose primer sequences
- GC = 30-60%
- 20bp or longer
- gel-separable amplification products
Step 2. Test/align primer sequences
- with each other
- BLAST programs for repetitive seq.
Step 3. Single locus PCR: initial program
- amplify all loci individually, same conditions
- take 1x PCR buffer, no adjuvants,
- adjust cycling conditions only
Step 4. Multiplex PCR: equimolar primer mix
- 0.1-0.4µM each primer
- use PCR program from Step 3
A. All products are weak
-a) use longer extension times
-b) decrease extension t° to 62-68° C
-c) decrease annealing t° in small steps (2°C)
-d) adjust Taq polymerase concentration
-e) combine Aa, Ab, Ac and Ad
B. Short products are weak
-a) increase buffer conc. from 1x to 1.4-2x
-b) decrease annealing and/or extension t°
-c) increase amount of primer for weak loci
-d) combine Ba, Bb, Bc
C. Long products are weak
-a) increase extension time
-b) increase annealing and/or extension t°
-c) increase amount of primer for weak loci
-d) decrease buffer conc. to 0.7-0.8x
but keep MgCl2 constant at 1.5-2mM
-e) combine Ca, Cb, Cc, Cd
D. Unspecific products appear
-a) if long: increase buffer conc. to 1.4-2.0x
-b) if short: decrease buffer conc. to 0.7-09x
-c) increase gradually annealing t°
-d) decrease amount of template and enzyme
-e) increase MgCl2 to 3, 6, 9 and 12mM but
keep dNTP constant at 200 µM each
-f) combine Da, Db, Dc, Dd, De
E. If none of the above works
-a) use adjuvants: try BSA (0.1-0.8µg/µl)
-b) use adjuvants: try 5%(w/v) DMSO or glycerol
-c) re-check primers for interactions with each other
-d) change all solutions, use fresh dNTP
Step 5

Rapid Detection and Identification of Probiotic *Lactobacillus casei* ATCC 393 by Multiplex PCR

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The *hsp60* gene sequences of all 56 *Lactobacillus* strains available in GenBank, including the *L. casei* ATCC 393 strain, were obtained by the National Center for Biotechnology Information (NCBI). The sequences were aligned against each other using the DNA sequence alignment tool, ClustalW2 (European Bioinformatics Institute – EMBL). Extensive analysis allowed the identification of polymorphic sites that are unique for the *L. casei* ATCC 393 strain.

Table 1. List of *Lactobacillus* strains included in this study

Reference strains	Collection No.	Accession No.	Bases at diagnostic sites (78, 156, 189)
Lactobacillus acetotolerans	**DSM 20749**	**AF429666**	**A-T-T**
Lactobacillus acidipiscis	DSM 15836	AJ621719	T-A-T
Lactobacillus acidophilus	DSM 20079	AY424311	T-A-T
Lactobacillus alimentarius	DSM 20249	AY424318	T-A-T
Lactobacillus amylophilus	DSM 20533	AY424319	G-A-C
Lactobacillus amylovorus	DSM 20531	AY424312	C-A-T
Lactobacillus bifermentans	DSM 20003	AY424320	T-T-C
Lactobacillus brevis	DSM 20054	AY424329	T-A-C
Lactobacillus buchneri	DSM 20057	AY571676	T-C-A
Lactobacillus casei	**ATCC 393**	**AY424336**	**G-C-T**
Lactobacillus casei	**ATCC 11578**	**n.a.**	**n.a.**
Lactobacillus casei	**DN114001**	**n.a.**	**n.a.**
Lactobacillus casei	**BB-12**	**n.a.**	**n.a.**
Lactobacillus casei	CCC B1241	AF429642	C-A-C
Lactobacillus casei	DSA-FB2	AY424335	C-A-C
Lactobacillus casei	ATCC 4913	AF429654	T-A-C
Lactobacillus casei	CCC B1205	AF429631	C-A-C
Lactobacillus casei	DSA 145	AY424333	C-A-C
Lactobacillus casei	ATCC 334	AY424332	C-A-C
Lactobacillus casei	CCC 95G2L	AF429701	C-A-C
Lactobacillus casei	DSA 15	AY424334	C-A-C
Lactobacillus casei	I18C	AF429649	C-A-C
Lactobacillus casei	CCC B9657	AF429644	C-A-C
Lactobacillus casei	I03	AF429647	C-A-C
Lactobacillus coryniformis	DSM 20001	AY424321	C-T-T
Lactobacillus crispatus	DSM 20584	AY424313	T-T-T
Lactobacillus curvatus	DSA 32Y	AY424348	T-T-A
Lactobacillus cypricasei	DSM 15353	AJ621720	T-A-T
Lactobacillus delbrueckii subsp. lactis	DSM 20072	AY424322	C-T-C
Lactobacillus durianis	DSM 15802	AJ621721	T-T-T
Lactobacillus farciminis	DSM 20184	AY424323	A-T-C
Lactobacillus fermentum	DSA FB5	AY424325	C-T-C
Lactobacillus fructivorans	DSM 20203	AF429681	C-C-A
Lactobacillus gallinarum	DSM 10532	AY571675	T-T-T
Lactobacillus gasseri	DSM 20243	AY424314	T-T-A
Lactobacillus helveticus	DSM 20075	AY424315	T-T-T
Lactobacillus hilgardii	DSM 20571	AF429699	T-T-A
Lactobacillus homohiochii	ATCC 15434	AF429685	T-T-T

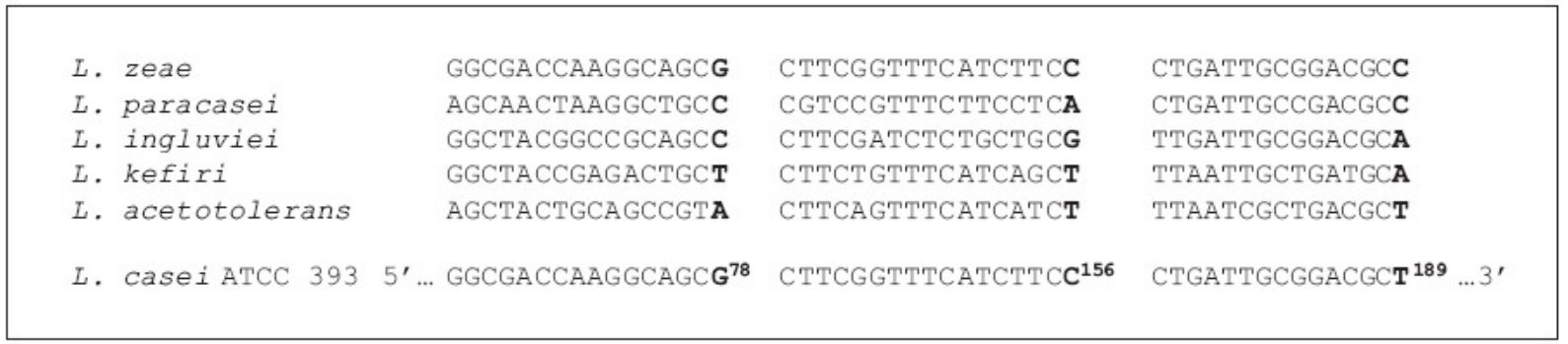

Fig. 1. The *hsp60* gene sequence containing the diagnostic nucleotides (in bold) targeted by the novel primers designed in this study.

Table 2. Oligonucleotide primers used in this study

Primer	Primer sequence (5′→3′)	Length bp	Optimal amount pmol
p78F	GGCGACCAAGGCAGCG	16	10
p156F	CTTCGGTTTCATCTTCC	17	50
p189R	GGCCAACTTTTTCCATA	17	50
LacF	AGCAGTAGGGAATCTTCCA	19	10
LacR	ATTYCACCGCTACACATG	18	10

Table 3. Specificity of primers designed for multiplex PCR on *Lactobacillus* strains

Reference strain	Specificity of primer pairs		
	p78F/ p189R (144 bp)	p156F/ p189R (67 bp)	LacF/ LacR (340 bp)
L. casei ATCC 393	+	+	+
L. acetotolerans DSM 20749	–	–	+
L. ingluviei DSM 15946	–	–	+
L. paracasei DSM 5622	–	–	+
L. zeae DSM 20178	–	–	+

The expected size of PCR products is indicated.

Optimization of Multiplex PCR

- •Cycling Conditions
- •Extension temperature
- •Extension time
- •Annealing time and temperature
- •Number of PCR cycles

Optimization of Multiplex Reaction Components

- •Amount of primer
- •dNTP and MgCl2 concentrations
- •PCR buffer (KCl) concentration.
- •Comparison of PCR buffers
- •Amount of template DNA and *Taq* DNA polymerase
- •Use of adjuvants: DMSO, glycerol, BSA

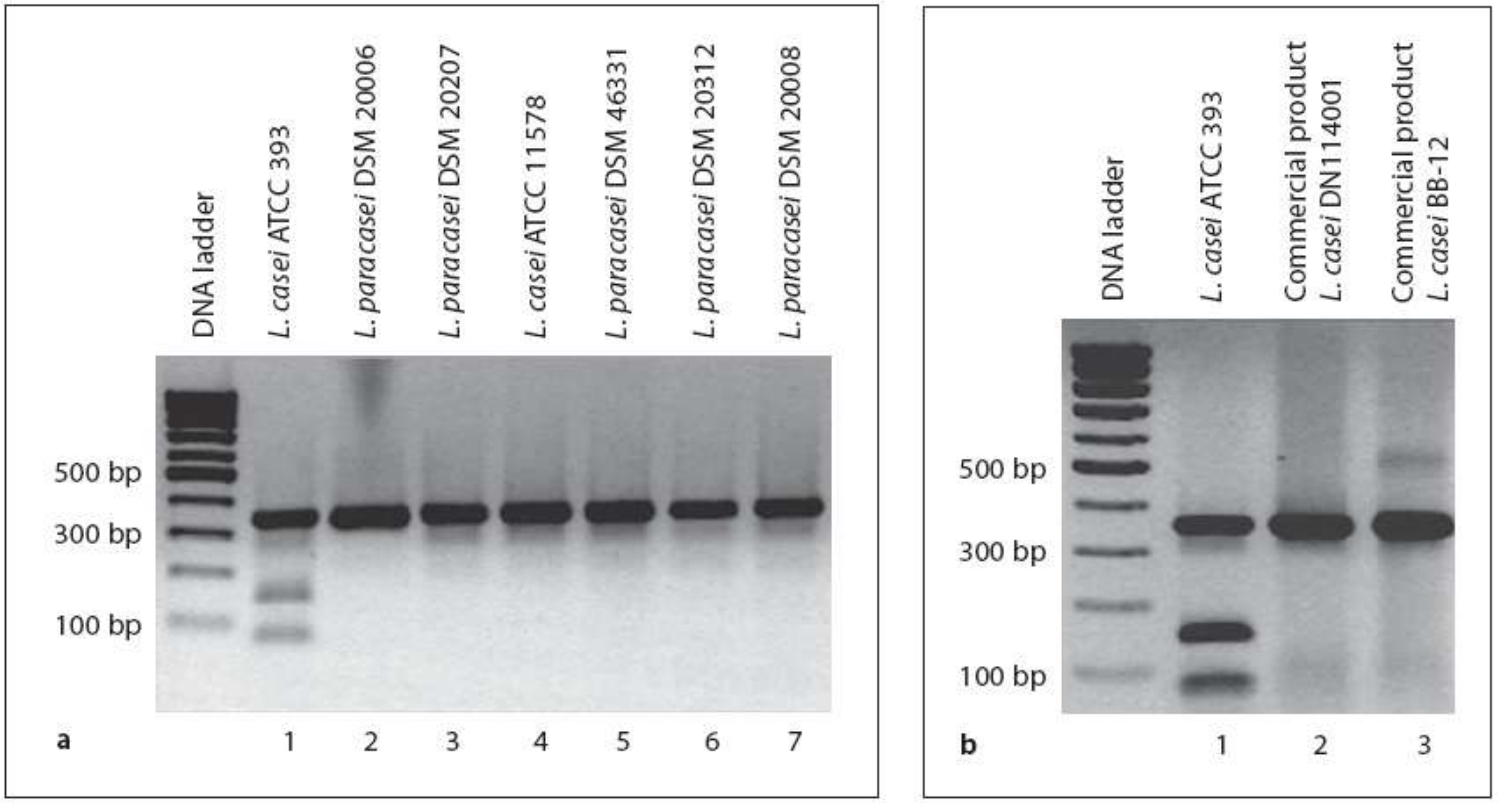

Fig. 3. Multiplex PCR assay of *L. casei* and *L. paracasei* strains (**a**) and two commercial probiotic products containing *L. casei* DN114001 and *L. casei* BB-12 (**b**).

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Note

A new methodology for rapid detection of *Lactobacillus delbrueckii* subsp. *bulgaricus* based on multiplex PCR

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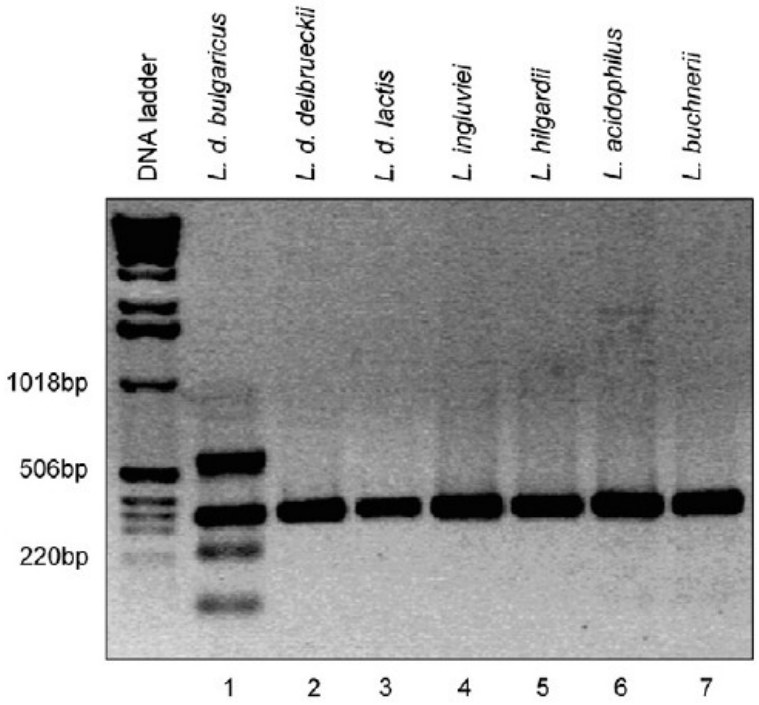
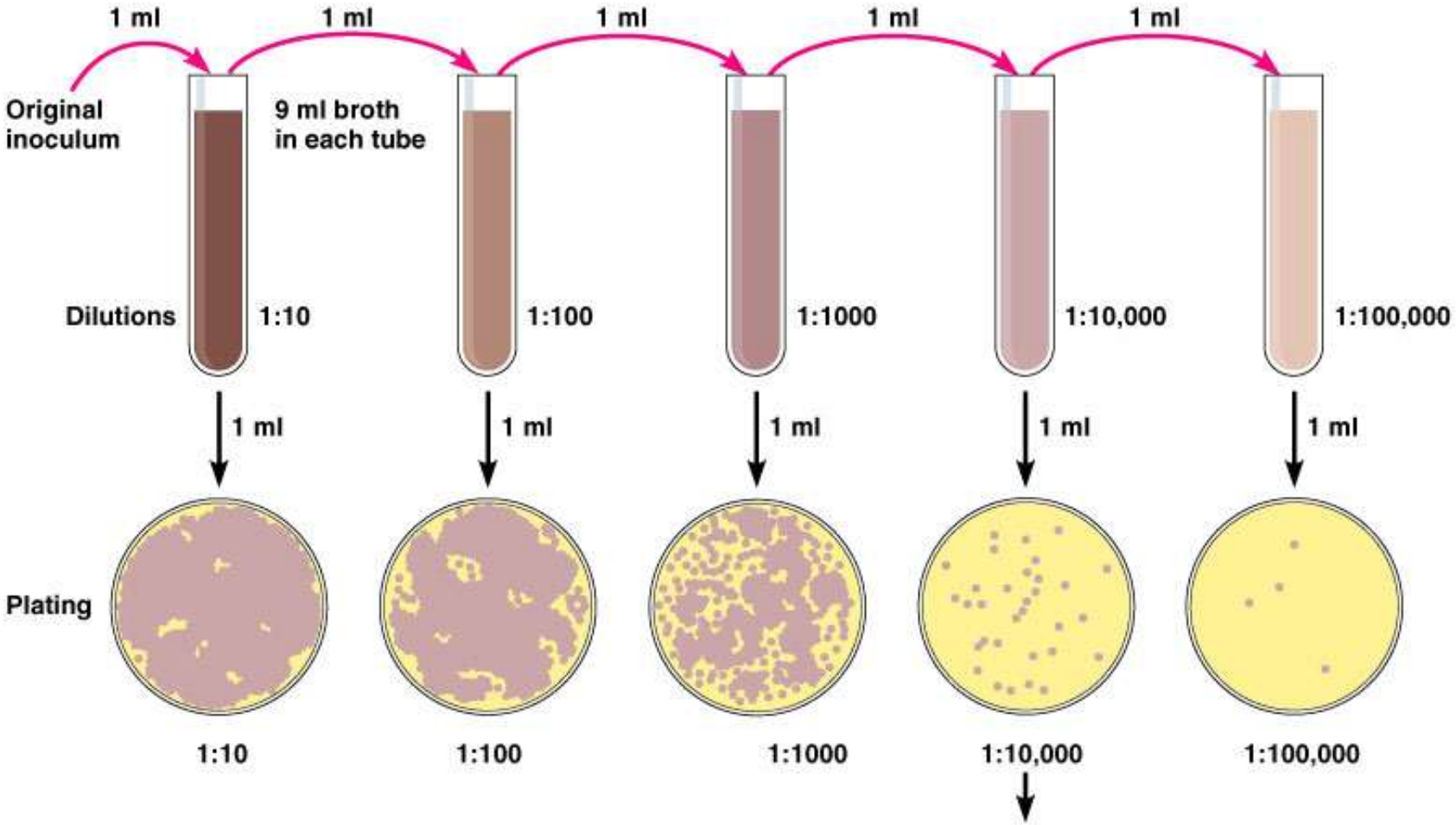


- The multiplex PCR methodology is an efficient tool for accurate, convenient and reliable identification of the probiotic strain in food products.

- To deliver their health benefits, probiotics must be present in food products above a threshold level (6 logCFU/g) at the time of consumption, to compensate for the loss of cells during the passage through the upper and lower parts of the gastrointestinal (GI) tract.

- Identification to the strain level is mandatory to allow the monitoring of the presence and the levels of a specific probiotic strain in a product or in a gastrointestinal tract.



Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000/ml in sample.)

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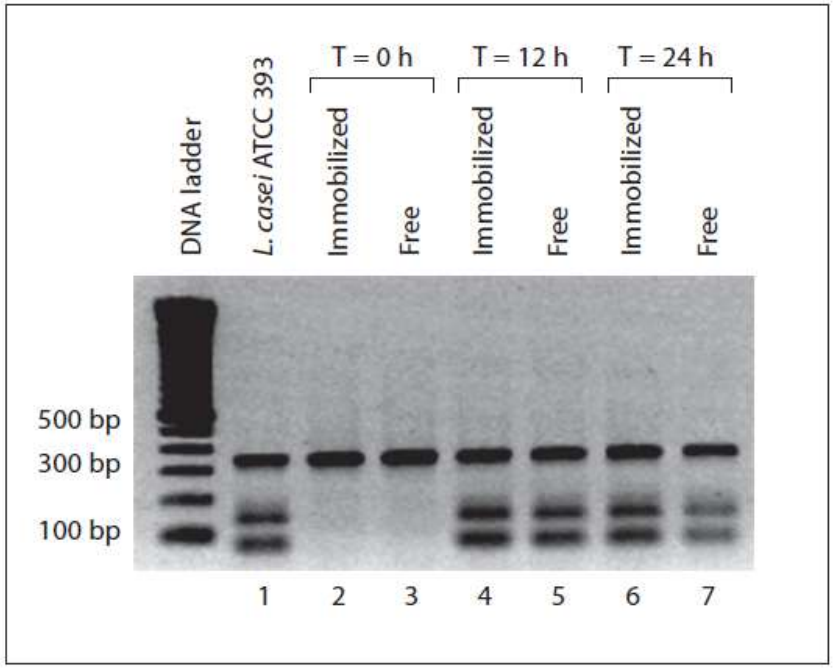
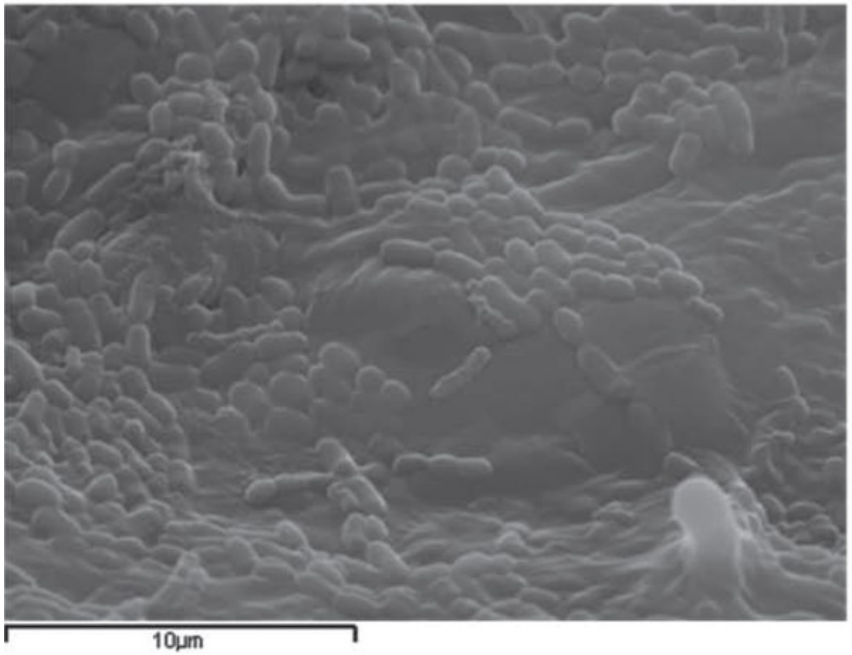
Effect of Probiotic-Fermented Milk Administration on Gastrointestinal Survival of *Lactobacillus casei* ATCC 393 and Modulation of Intestinal Microbial Flora

Marianthi Sidira[a] Alex Galanis[a] Petros Ypsilantis[b] Athanasios Karapetsas[a]
Zoi Progaki[a] Constantinos Simopoulos[b] Yiannis Kourkoutas[a]





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Anaerobe

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Distinct adhesion of probiotic strain *Lactobacillus casei* ATCC 393 to rat intestinal mucosa

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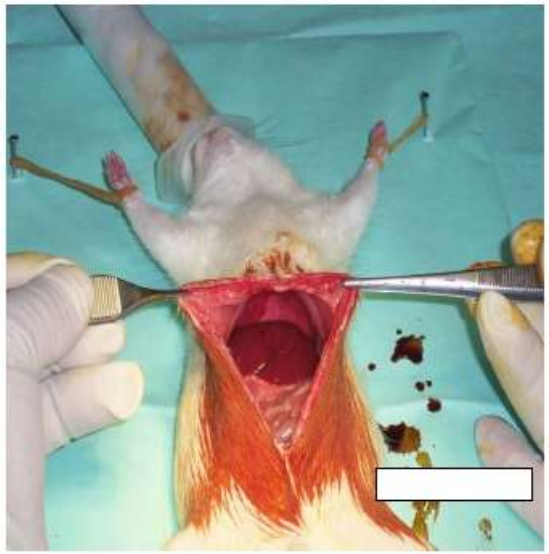
Levels of *L. casei* ATCC 393 recovered from intestinal content and tissue samples of the GI tract of rats 24 h after single dose administration of fermented milk containing either free (Group F1–24) or immobilized (Group I1–24) probiotic bacteria.

Specimens	Group F1–24 (logCFU/g)	Group I1–24 (logCFU/g)
Duodenal fluid	ND	ND
Jejunal fluid	3	3
Ileal fluid	3	3
Cecal fluid	5	6
Colon fluid	5	6
Duodenum	ND	ND
Jejunum	3	3
Ileum	3	3
Cecum	5	5
Colon	5	5

ND: not detected.



J. Dairy Sci. 96:1–9
http://dx.doi.org/10.3168/jds.2012-6343

Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool

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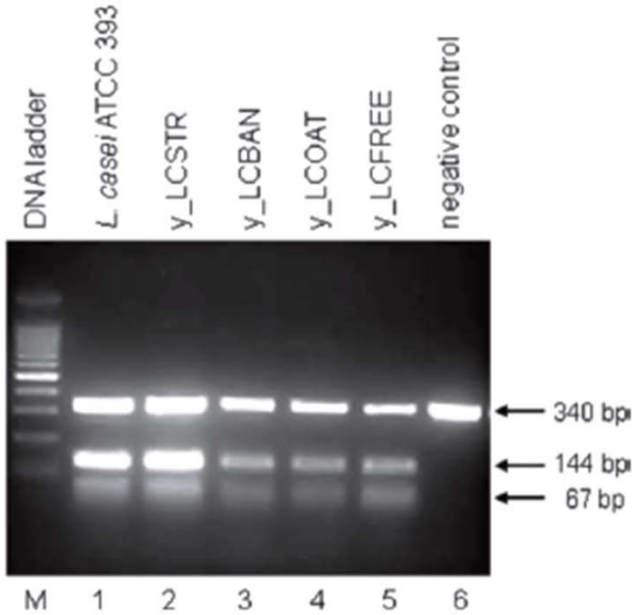
†Food Biotechnology Group, Section of Analytical Environmental and Applied Chemistry, Department of Chemistry, University of Patras, GR-26500 Patras, Greece



Στη περίπτωση που το γονιδίωμα των μικροοργανισμών που πρόκειται να μελετηθούν δεν είναι γνωστό μπορεί να χρησιμοποιηθεί η τεχνική RAPD PCR (Τυχαία Ενίσχυση Πολυμορφικού DNA PCR ή Random Amplified Polymorphic DNA PCR). Σύμφωνα με την εν λόγω τεχνική, η αντίδραση PCR πραγματοποιείται με εκκινητές τυχαίας αλληλουχίας, μήκους 8-12 βάσεων. Συνήθως απαιτούνται 3 με 6 αντιδράσεις με διαφορετικούς εκκινητές ώστε να προκύψει ένα μοναδικό πρότυπο για κάθε εξεταζόμενο οργανισμό, μετά το διαχωρισμό σε πήκτωμα αγαρόζης των προϊόντων της αντίδρασης PCR. Στα πλεονεκτήματα της μεθόδου περιλαμβάνονται η υψηλή ακρίβεια της ανάλυσης, ο χαμηλός χρόνος ολοκλήρωσης της μελέτης καθώς και το σχετικά χαμηλό κόστος των αντιδραστηρίων. Η RAPD PCR έχει χρησιμοποιηθεί για την ανίχνευση και ταυτοποίηση διαφόρων λακτοβακίλων σε καλλιέργειες γιαούρτης καθώς και σε άλλα προβιοτικά τρόφιμα.

Random Amplified Polymorphic DNA (RAPD)

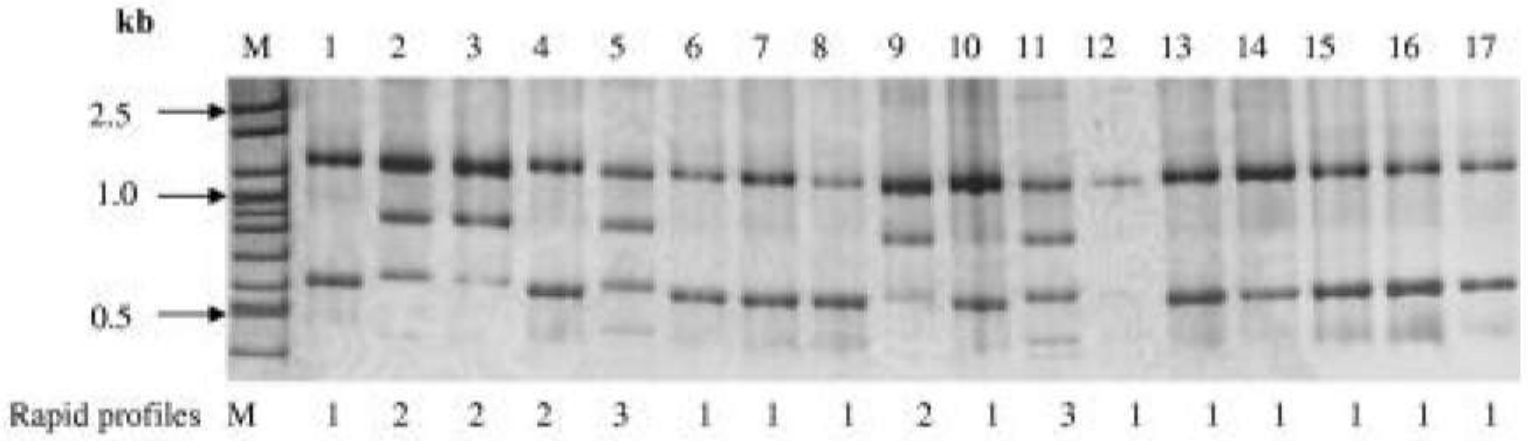
Introduction

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence.

How It Works

Unlike traditional PCR analysis, RAPD (pronounced "rapid") does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

RAPD is an inexpensive yet powerful typing method for many bacterial species.

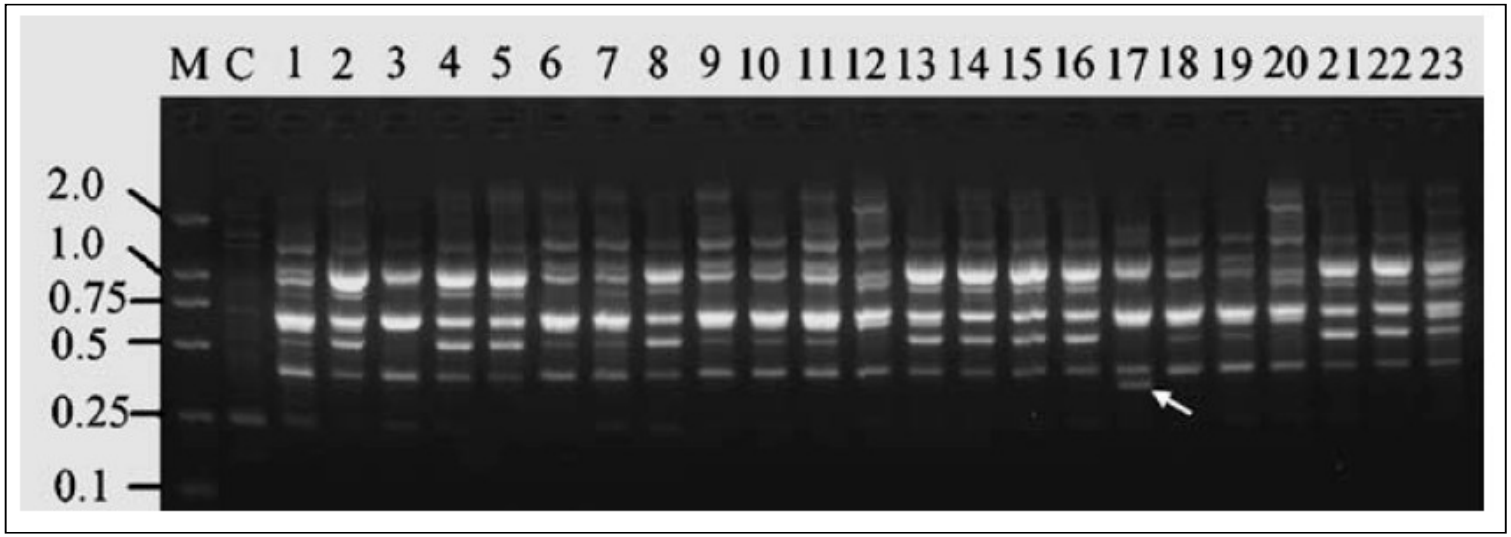


Silver-stained polyacrylamide gel showing three distinct RAPD profiles generated by primer OPE15 for *Haemophilus ducreyi* isolates from Tanzania, Senegal, Thailand, Europe, and North America.

Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains.

Sequenced Characterized Amplified Region (SCAR)

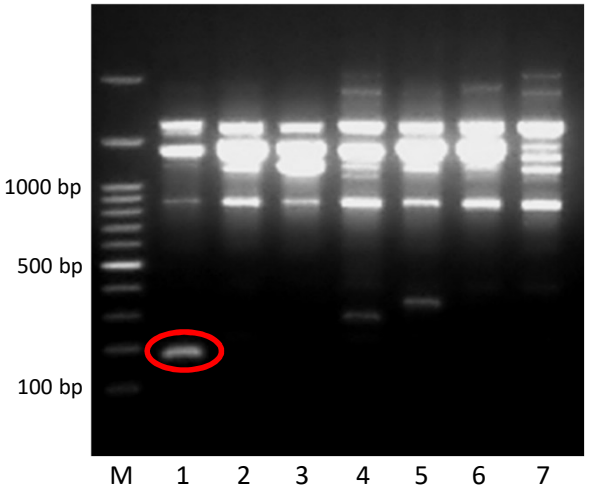
Τυχαία Ενίσχυση Πολυμορφικού DNA



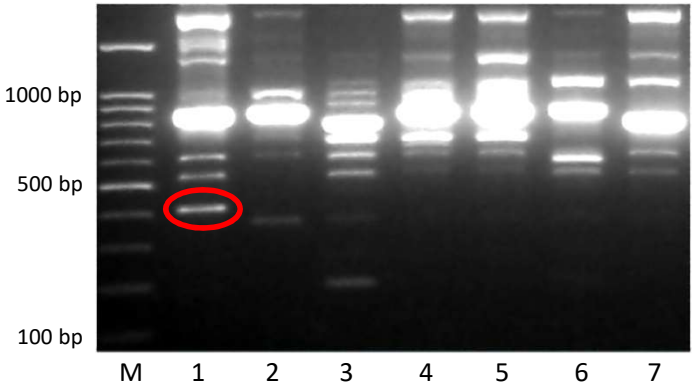
1. Απομόνωση από το πήκτωμα αγαρόζης της πολυμορφικής ζώνης
2. Ενίσχυση με αντίδραση PCR
3. Αλληλούχιση του προϊόντος PCR
4. Σχεδιασμός εκκινητών βάση της DNA αλληλουχίας του προϊόντος PCR
5. Ανάπτυξη Multiplex PCR

Li et al., (2008) Appl. Micro. Biotech.

Lactobacillus plantarum 2035



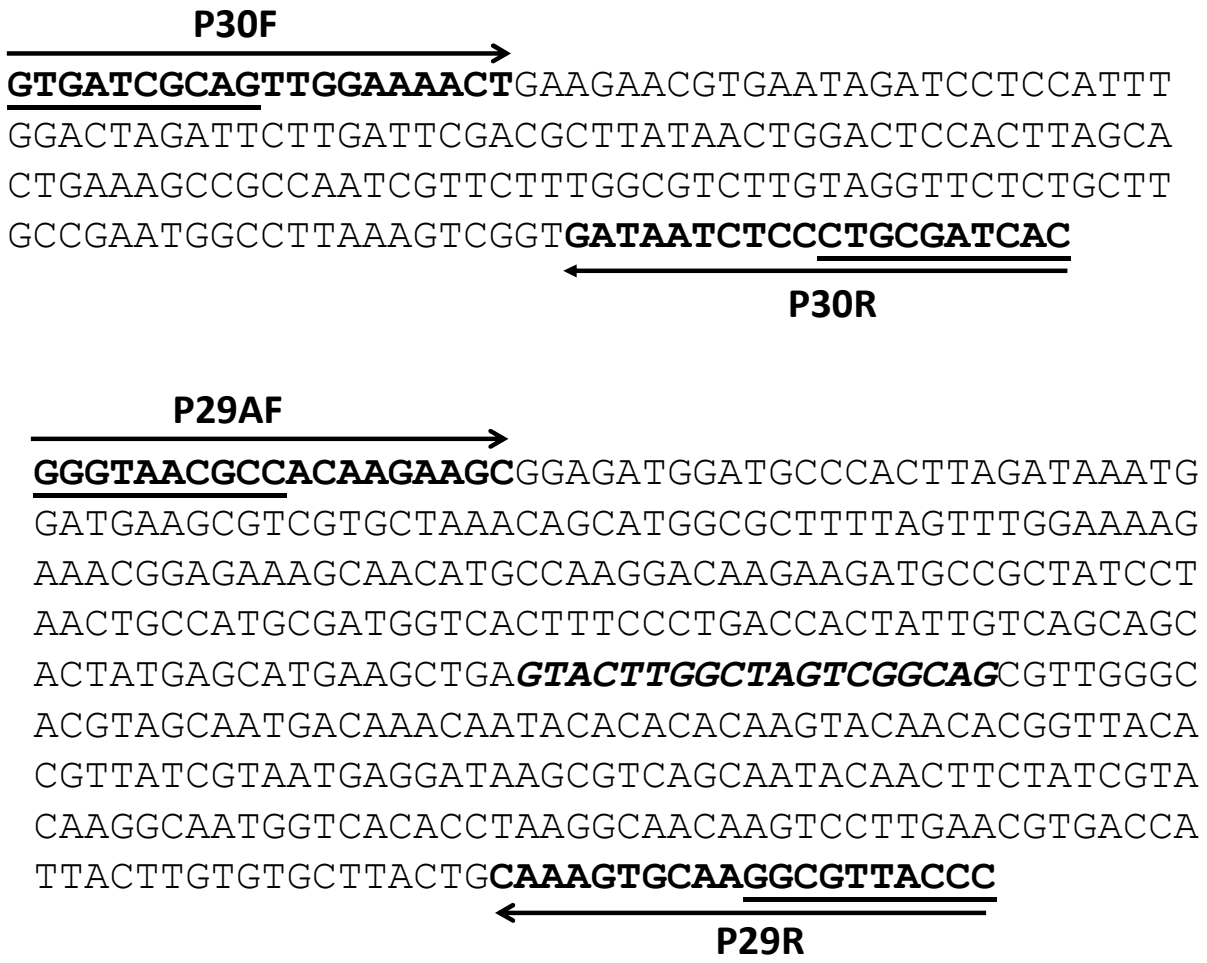
Lactobacillus plantarum 2640



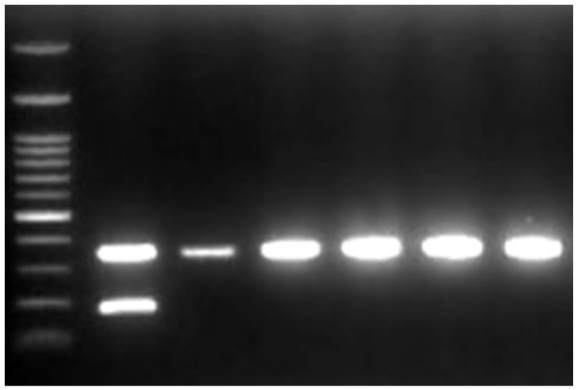
Sequenced Characterized Amplified Region Markers (SCARs)

Developing locus-specific, co-dominant markers from RAPDs

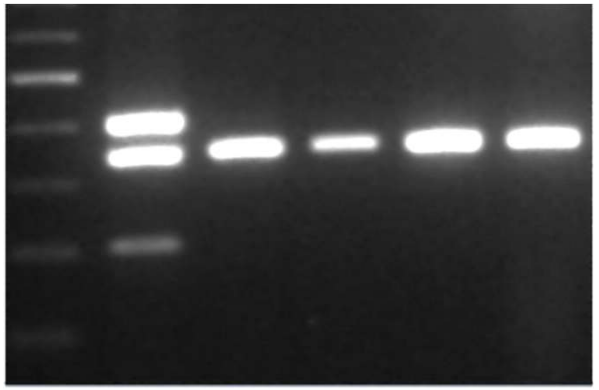
1. The polymorphic RAPD marker band is isolated from the gel.
2. It is amplified in the PCR reaction.
3. The PCR product is cloned and sequenced.
4. New longer and specific primers are designed for the DNA sequence, which is called the Sequenced Characterized Amplified Region Marker (SCAR).


P30F
GTGATCGCAGTTGGAAAACTGAAGAACGTGAATAGATCCTCCATTT
GGACTAGATTCTTGATTCGACGCTTATAACTGGACTCCACTTAGCA
CTGAAAGCCGCCAATCGTTCTTTGGCGTCTTGTAGGTTCTCTGCTT
GCCGAATGGCCTTAAAGTCGGTGATAATCTCCCTGCGATCAC
P30R
P29AF
GGGTAACGCCACAAGAAGCGGAGATGGATGCCCACTTAGATAAATG
GATGAAGCGTCGTGCTAAACAGCATGGCGCTTTTAGTTTGGAAAAG
AAACGGAGAAAGCAACATGCCAAGGACAAGAAGATGCCGCTATCCT
AACTGCCATGCGATGGTCACTTTCCCTGACCACTATTGTCAGCAGC
ACTATGAGCATGAAGCTGAGTACTTGGCTAGTCGGCAGCGTTGGGC
ACGTAGCAATGACAAACAATACACACACAAGTACAACACGGTTACA
CGTTATCGTAATGAGGATAAGCGTCAGCAATACAACTTCTATCGTA
CAAGGCAATGGTCACACCTAAGGCAACAAGTCCTTGAACGTGACCA
TTACTTGTGTGCTTACTGCAAAGTGCAAGGCGTTACCC
P29R

Lactobacillus plantarum 2035



Lactobacillus plantarum 2640

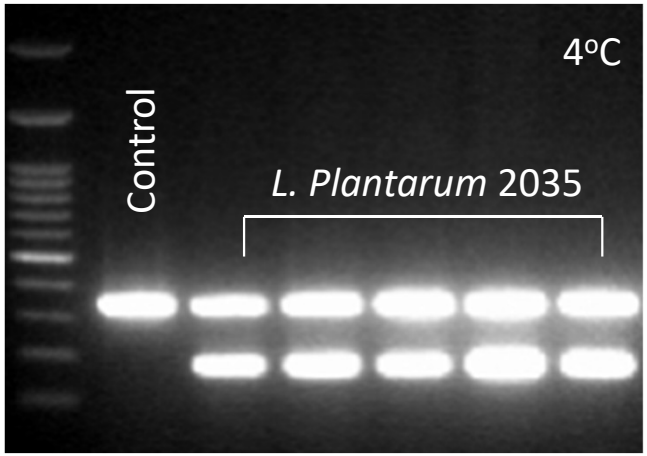


Reference strain	**Specificity of primer pairs**			
	p30F/p30R (179bp)	p29AF/p29R (401bp)	p29BF/p29R (200bp)	LacF/LacR (340bp)
Lb. plantarum 2035	+	-	-	+
Lb. plantarum 2640	-	+	+	+

Strain tested	**2-band pattern generated with Multiplex PCR**
***Lactobacillus plantarum* 2035**	+
Lb. plantarum 2640	-
Lb. plantarum E1	-
Lb. plantarum E4	-
Lb. plantarum E45	-
Lb. plantarum E50	-
Lb. plantarum E66	-
Lb. plantarum E68	-
Lb. plantarum E71	-
Lb. plantarum E73	-
Lb. plantarum E77	-
Lb. plantarum E79	-
Lb. plantarum E10	-
Lb. plantarum E69	-
Lb. plantarum E282	-
Lb. plantarum E63	-
Lb. plantarum E146	-
Lb. plantarum E287	-
Lb. pentosus E110	-
Lb. pentosus 281	-
Lb. casei Shirota ACA-DC 6002	-
Lb. casei ATCC 393	-
Lb. rhamnosus GG ATCC 53103	-

Strain tested	**3-band pattern with Multiplex PCR**
***Lactobacillus plantarum* 2640**	+
Lb. plantarum 2035	-
Lb. plantarum E1	-
Lb. plantarum E4	-
Lb. plantarum E45	-
Lb. plantarum E50	-
Lb. plantarum E66	-
Lb. plantarum E68	-
Lb. plantarum E71	-
Lb. plantarum E73	-
Lb. plantarum E77	-
Lb. plantarum E79	-
Lb. plantarum E10	-
Lb. plantarum E69	-
Lb. plantarum E282	-
Lb. plantarum E63	-
Lb. plantarum E146	-
Lb. plantarum E287	-
Lb. pentosus E110	-
Lb. pentosus 281	-
Lb. casei Shirota ACA-DC 6002	-
Lb. casei ATCC 393	-
Lb. rhamnosus GG ATCC 53103	-

Production of Fermented Sausages with a Potential Probiotic *Lactobacillus plantarum* strain



Production of Greek yoghurt using a *L. plantarum* strain with probiotic potential as starter adjunct

ProbioDairyMeat

Родопи

Production of Greek yoghurt using a *L. plantarum* strain with probiotic potential as starter adjunct

ProbioDairyMeat

Ροδοπη

Η PCR πραγματικού χρόνου (Real-Time PCR) επίσης αποτελεί μια πολύ ισχυρή μοριακή τεχνική ανίχνευσης μικροοργανισμών. Βασίζεται στη καταγραφή της ενίσχυσης του DNA-στόχου σε πραγματικό χρόνο με τη χρήση κατάλληλης φθορίζουσας χρωστικής. Έτσι, γίνεται άμεση καταγραφή του αποτελέσματος χωρίς να απαιτείται περαιτέρω επεξεργασία των προϊόντων της αντίδρασης (διαχωρισμός σε πήκτωμα αγαρόζης). Παράλληλα, το δυναμικό εύρος διάγνωσης και η ευαισθησία της μεθόδου είναι υψηλότερα ενώ τα ψευδώς θετικά αποτελέσματα που ενδεχομένως να παρατηρηθούν σε σχέση με τη συμβατική PCR είναι μειωμένα κατά 99.9%. Η Real-Time PCR έχει χρησιμοποιηθεί για την μοριακή ταυτοποίηση και ποσοτικοποίηση των *L. casei, L. paracasei, L. rhamnosus, L. delbrueckii, L. fermentum, L.plantarum* και *L. reuteri* με χρήση ανιχνευτών ειδικών για τμήμα του ριβοσωμικού γονιδίου 16S αυτών των στελεχών αλλά και με ανάλογο τρόπο για την ταυτοποίηση στελεχών *L. acidophilus*.

Οι **DGGE (Denaturing Gradient Gel Electrophoresis - ηλεκτροφόρηση πηκτώματος σε διαβάθμιση αποδιατακτικού παράγοντα) και TGGE (Temperature Gradient Gel Electrophoresis ηλεκτροφόρηση πηκτώματος σε διαβάθμιση θερμοκρασίας)** είναι PCR βασιζόμενες μοριακές τεχνικές που επιτρέπουν την ανάλυση και ταυτοποίηση βακτηριακών στελεχών ετερόλογων πληθυσμών, χωρίς να προαπαιτείται διαχωρισμός των μικροβιακών καλλιεργειών. Για παράδειγμα, η τεχνική DGGE έχει με επιτυχία χρησιμοποιηθεί για την ανίχνευση στελεχών *L. delbrueckii* subsp. *bulgaricus* και *S. thermophilus* σε ανθρώπινο εντερικό περιεχόμενο ύστερα από κατανάλωση προβιοτικής γιαούρτης, γεγονός ιδιαίτερα σημαντικό για την επιβεβαίωση της δράση του προβιοτικού τροφίμου. Παρόμοιες μελέτες έχουν αναφερθεί και για τη χρήση της τεχνικής TGGE.

Η DGGE (Denaturing Gradient Gel Electrophoresis) (Ηλεκτροφόρηση πηκτώματος διαβαθμισμένης συγκέντρωσης παράγοντα αποδιάταξης) είναι μια μοριακή τεχνική ανίχνευσης πολυμορφισμών τμημάτων DNA. Η τεχνική είναι ιδιαιτέρα ισχυρή και αποτελεσματική για την ανίχνευση μεταλλάξεων σε γονίδια αλλά και ταυτοποίηση μικροοργανισμών που παρουσιάζουν υψηλό βαθμό συγγένειας. Η ανίχνευση πολυμορφισμών τμημάτων DNA είναι επίσης εφικτή με την αντίδραση PCR (polymerase chain reaction) (αλυσιδωτή αντίδραση πολυμεράσης) χρησιμοποιώντας εξειδικευμένους εκκινητές. Στις περιπτώσεις όμως που τα προϊόντα της PCR είναι παρόμοιου μεγέθους, ο διαχωρισμός τους σε πήκτωμα αγαρόζης καθίσταται τεχνικά μη εφικτός. Αντίθετα, στην τεχνική DGGE, ο διαχωρισμός των τμημάτων DNA γίνεται σε πήκτωμα πολυακριλαμίδης διαβαθμισμένης συγκέντρωσης αποδιατακτικού παράγοντα που καθιστά το διαχωρισμό των τμημάτων εφικτό.

Πιο συγκεκριμένα ο διαχωρισμός αρχικά γίνεται βάση μεγέθους αλλά στη συνέχεια με την αύξηση της συγκέντρωσης του αποδιατακτικού παράγοντα του πηκτώματος το δίκλωνο DNA μετατρέπεται σε μονόκλωνο (αποδιατάσσεται) με αποτέλεσμα η ταχύτητα κίνησης του στο πήκτωμα πολυακριλαμίδης κατά την ηλεκτροφόρηση να μειώνεται. Έτσι ακόμα και δύο τμήματα DNA που διαφέρουν σε μία μόνο βάση αποδιατάσσονται σε διαφορετική συγκέντρωση, κινούνται διαφορετικά στο πήκτωμα και τελικά διαχωρίζονται. Η τεχνική DGGE έχει χρησιμοποιηθεί σε πολλές μελέτες σχετικές με τη βιοτεχνολογία τροφίμων καθώς δίνει τη δυνατότητα πλήρους ταυτοποίησης της μικροβιακής χλωρίδας σε δείγματα τροφίμων με μεγάλη ακρίβεια και ταχύτητα.

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Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Review article

PCR-DGGE fingerprinting: novel strategies for detection of microbes in food

Danilo Ercolini*

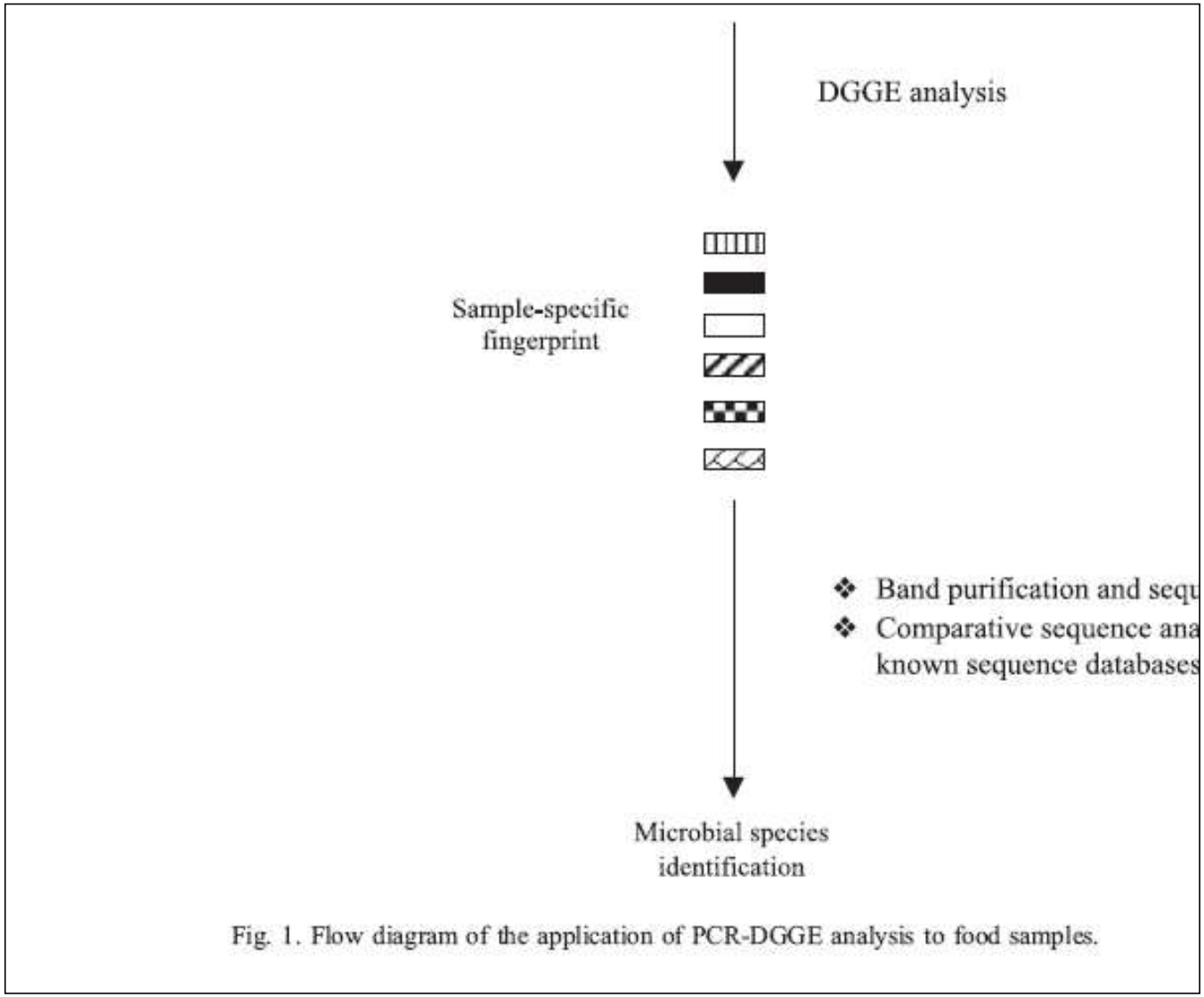
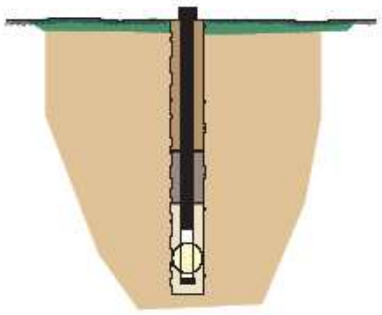


Fig. 1. Flow diagram of the application of PCR-DGGE analysis to food samples.

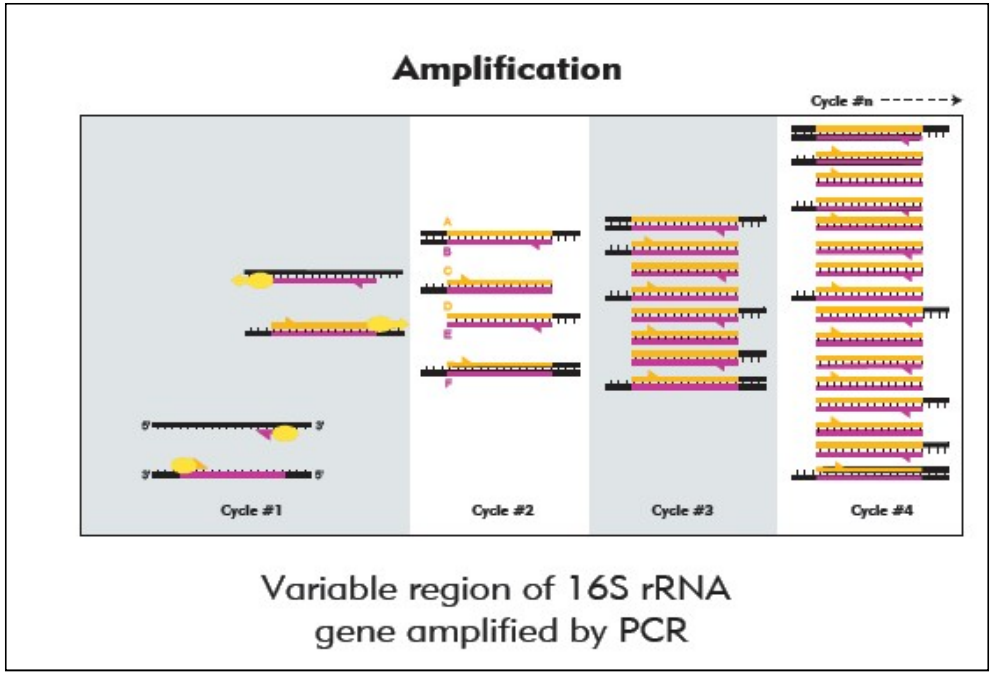
Sample Collection



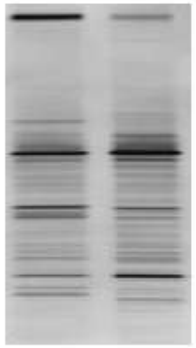
Groundwater, soil, or Bio-Trap® Sampler collected and shipped overnight on ice (4°c)

DNA Extraction

DNA is extracted from samples upon arrival


Amplification
Cycle #n
Cycle #1
Cycle #2
Cycle #3
Cycle #4
Variable region of 16S rRNA
gene amplified by PCR

Results



DGGE Profile

GCCAGCCGTGTTTACAGA…

GCCTTAAGCAGGCCTTCG…

GCCAGCCGTGTTTACAGA…

GCCGGCTCTAGCTTCCGT…

Dominant bands excised and sequenced

Acinetobacter sp.

Geobacter sp.

Unknown sp X.

Methylobacterium sp.

Dominant organisms identified

Denaturing Gradient Gel Electrophoresis (DGGE) is a DNA-based technique which generates a genetic profile or "fingerprint" which can be used to identify the dominant members of the microbial community. DGGE has been used to investigate microbial responses in a wide variety of applications including:

- Bioremediation assessment
- Wastewater treatment
- Drinking water treatment
- Biofilm formation
- Microbial induced corrosion
- Identification of microbial contaminants in commercial/industrial products

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2001, p. 5113–5121 Vol. 67, No. 11
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.11.5113–5121.2001

Denaturing Gradient Gel Electrophoresis Analysis of the 16S rRNA Gene V1 Region To Monitor Dynamic Changes in the Bacterial Population during Fermentation of Italian Sausages

LUCA COCOLIN,[1]* MARISA MANZANO,[1] CARLO CANTONI,[2] AND GIUSEPPE COMI[1]

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2004, p. 1883–1894 Vol. 70, No. 4
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.4.1883–1894.2004

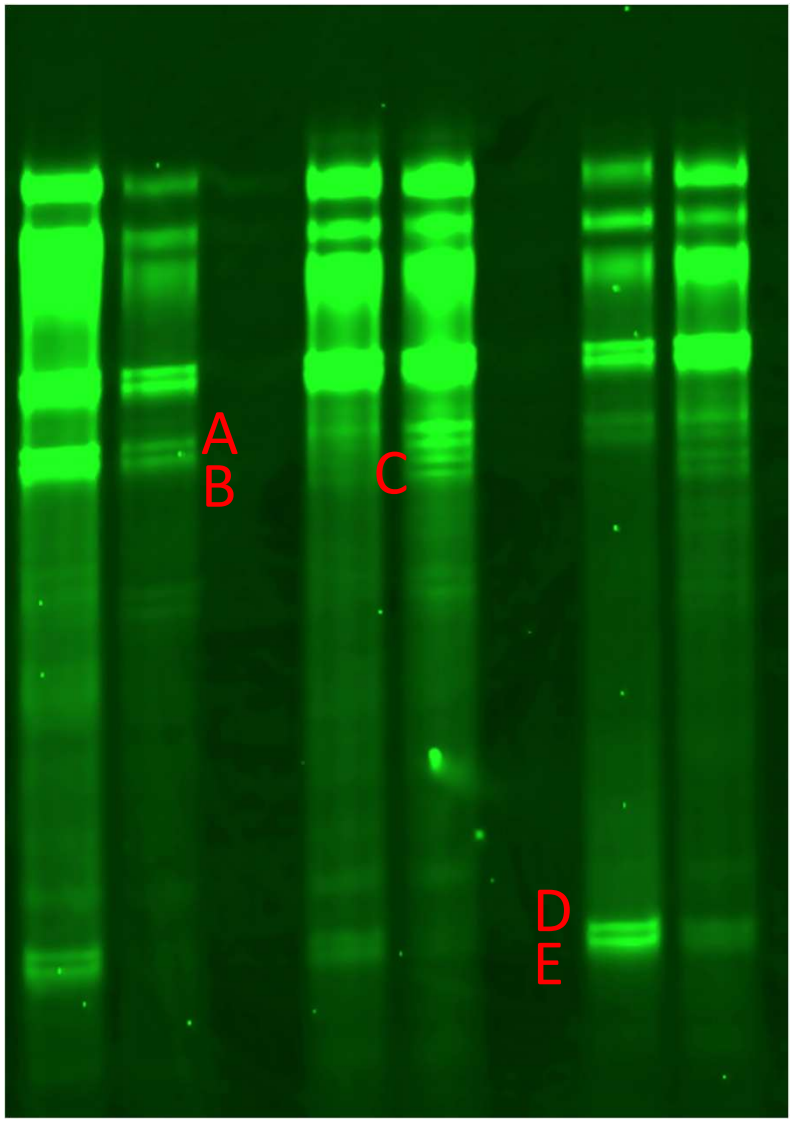
Study of the Ecology of Fresh Sausages and Characterization of Populations of Lactic Acid Bacteria by Molecular Methods

Luca Cocolin,[1]* Kalliopi Rantsiou,[1] Lucilla Iacumin,[1] Rosalinda Urso,[1] Carlo Cantoni,[2] and Giuseppe Comi[1]

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2003, p. 475–482 Vol. 69, No. 1
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.1.475–482.2003

Monitoring the Bacterial Population Dynamics in Sourdough Fermentation Processes by Using PCR-Denaturing Gradient Gel Electrophoresis

Christiane B. Meroth, Jens Walter, Christian Hertel,* Markus J. Brandt, and Walter P. Hammes



Στελέχη βακτηρίων που απομονώθηκαν από ξηρά ζυμωμένα λουκάνικα με ανάλυση PCR-DGGE

Band	Most closely related species
A	*Lactococcus lactis*
	Uncultured *Streptococcaceae* sp.
	Lactococcus fujiensis
B	*Lactococcus lactis*
	Uncultured bacterium
C	***Lactobacillus casei* ATCC 393**
	Lactobacillus zeae
	Uncultured *Lactobacillus* sp.
D	*Leuconostoc pseudomesenteroides*
	Leuconostoc citreum
	Leuconostoc mesenteroides
	Leuconostoc kimchii
E	*Leuconostoc* sp. B 244
	Leuconostoc mesenteroides

Meat Science 96 (2014) 948–955

Contents lists available at ScienceDirect

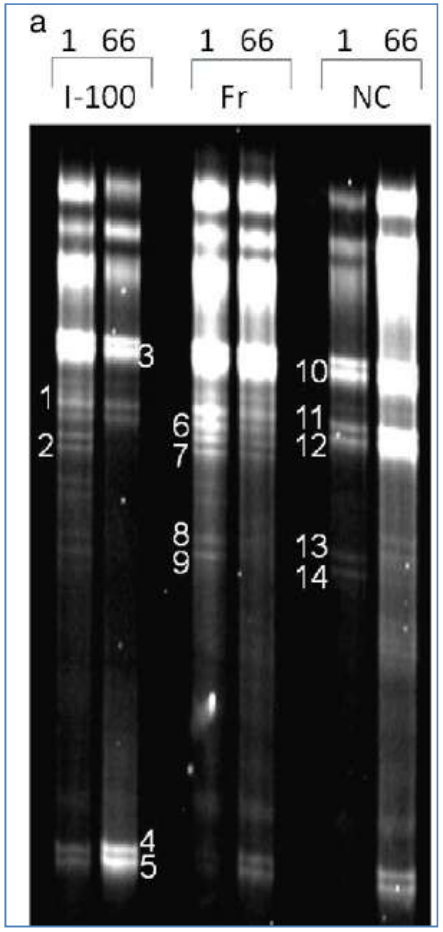
Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Effective survival of immobilized *Lactobacillus casei* during ripening and heat treatment of probiotic dry-fermented sausages and investigation of the microbial dynamics

Marianthi Sidira [a,b], Athanasios Karapetsas [b], Alex Galanis [b], Maria Kanellaki [a], Yiannis Kourkoutas [b,*]

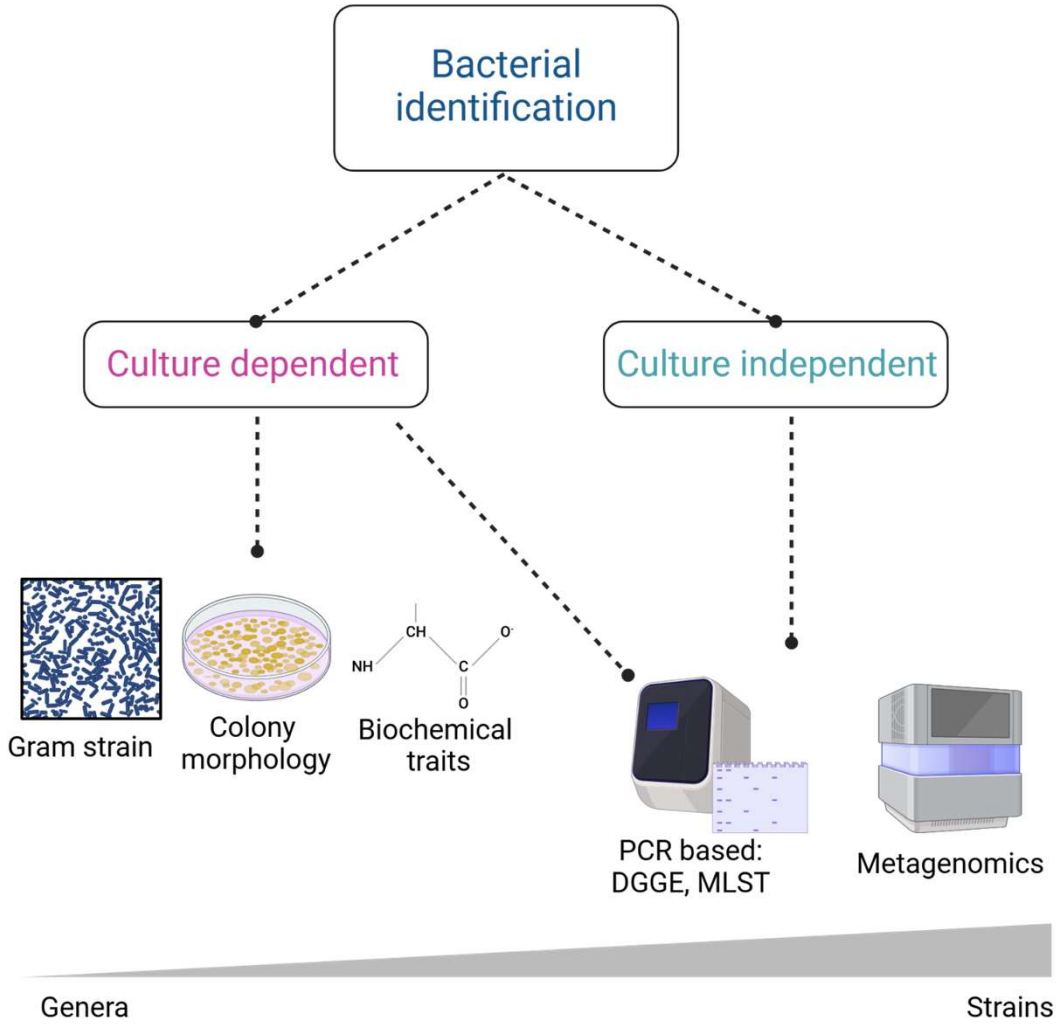
[a] Food Biotechnology Group, Section of Analytical Environmental and Applied Chemistry, Department of Chemistry, University of Patras, GR-26500 Patras, Greece.
[b] Applied Microbiology and Molecular Biotechnology Research Group, Department of Molecular Biology & Genetics, Democritus University of Thrace, Alexandroupolis 68100, Greece.



Phylogenetic affiliations of bacterial dynamics in probiotic dry-fermented sausages and sausages produced with no starter culture after 1 and 66 days of ripening based on DNA analyses and the corresponding band(s) in the DGGE profile.

Band[a]	Most closely related species	Identity (%)	Accession number[b]
1	*Bacillus thuringiensis*	98	JF512478.1
	Bacillus cereus	98	HQ683796.1
2	*Lactobacillus casei* ATCC 393	97	NR_041893.1
	Lactobacillus zeae	97	AB008212.1
3	*Brochothrix thermosphacta*	98	AF318169.1
4	*Leuconostoc pseudomesenteroides*	98	HE646416.1
	Leuconostoc citreum	98	AB602811.1
	Leuconostoc mesenteroides	98	AF318164.1
	Leuconostoc kimchii	98	CP001758.1
5	*Leuconostoc* sp. B 244	98	GU998869.1
	Leuconostoc mesenteroides	95	AF318164.1
6	*Lactococcus lactis* subsp. *cremoris*	96	AB681219.1
	Lactococcus lactis subsp. *lactis*	96	JF922121.1
7	*Lactobacillus casei* ATCC 393	97	NR_041893.1
	Lactobacillus zeae	97	AB008212.1
	Uncultured *Lactobacillus* sp.	97	AY857628.1
8	*Carnobacterium divergens*	98	AB598939.1
9	*Carnobacterium maltaromaticum*	96	GQ304931.1
10	*Brochothrix thermosphacta*	98	AF318169.1
11	*Lactococcus lactis*	100	AB571485.1
	Uncultured *Streptococcaceae* sp.	100	AB234513.1
	Lactococcus fujiensis	100	AB485959.1
12	*Lactococcus lactis*	100	EU091434.1
	Uncultured bacterium	98	JN884004.1
13	*Carnobacterium divergens*	98	AB598939.1
14	*Carnobacterium maltaromaticum*	96	GQ304931.1

Bacterial identification/detection methods

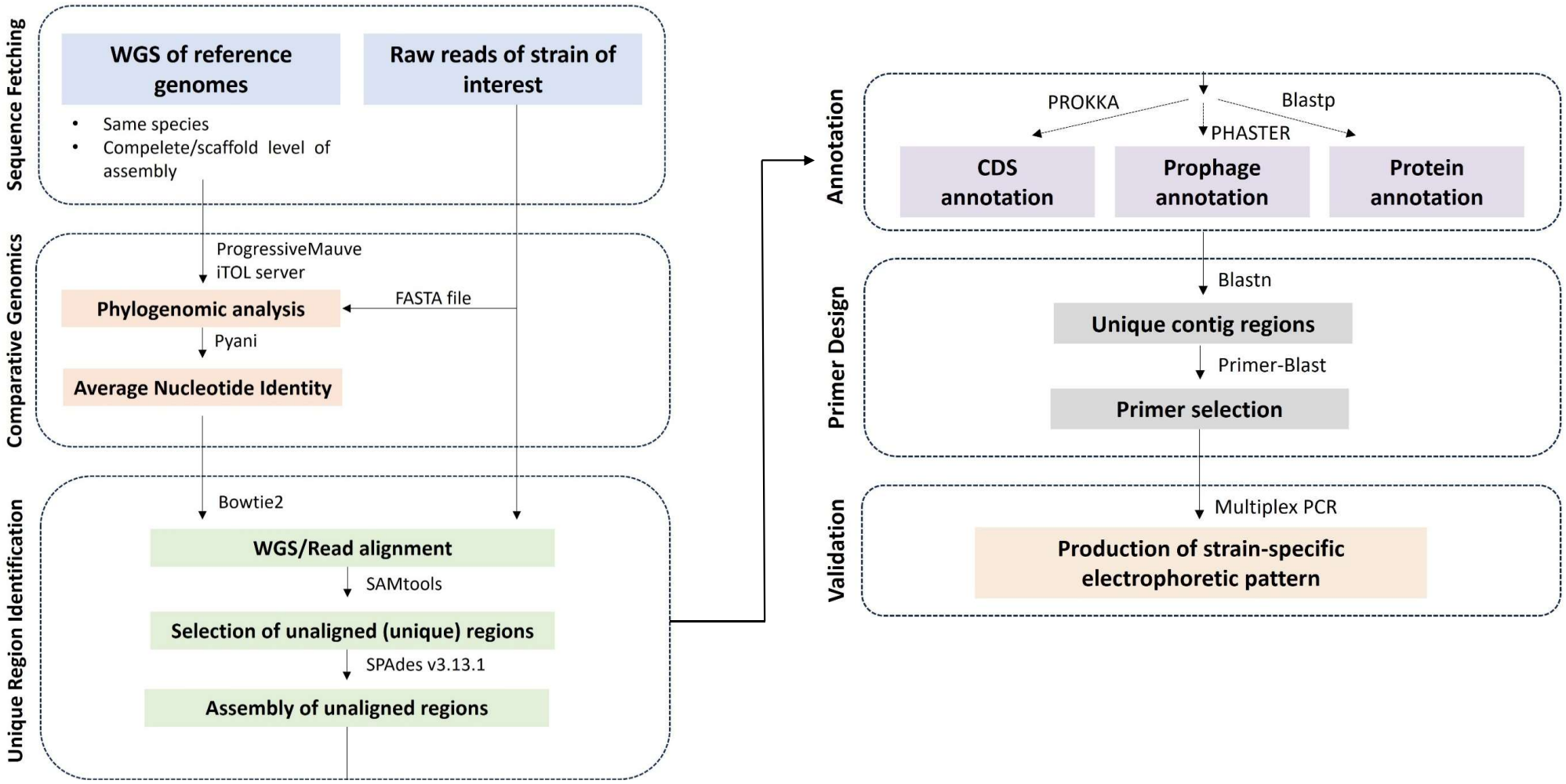


Development of a Multiplex PCR Assay for Efficient Detection of Two Potential Probiotic Strains Using Whole Genome-Based Primers

by **Despoina E. Kiousi** [1], **Dimitrios M. Karadedos** [1], **Anastasia Sykoudi** [1], **Panagiotis Repanas** [1], **Christina S. Kamarinou** [1,2], **Anthoula A. Argyri** [2] and **Alex Galanis** [1,*]

Microorganisms **2023**, *11*(10), 2553; **https://doi.org/10.3390/microorganisms11102553**

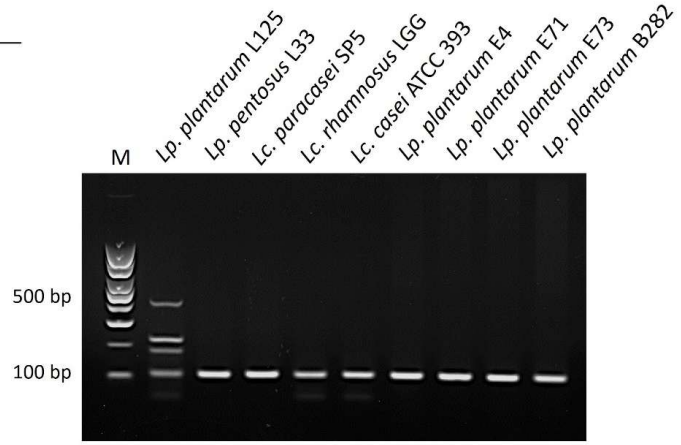
Schematic representation of the workflow



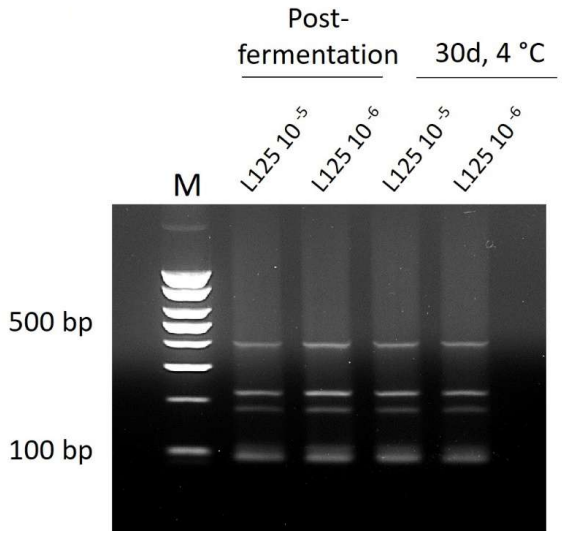
Validation of the methodology *in vitro*

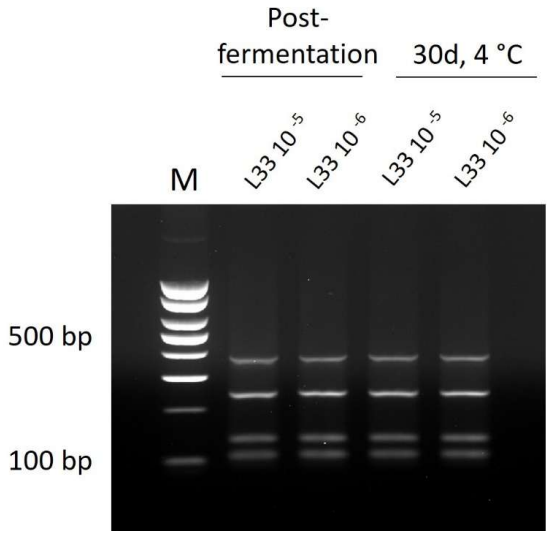
Monocultures

LAB strains	Specificity of primer sets			
	6.2 (183 bp)	10.16 (405 bp)	12.1 (223 bp)	P1/P2 (107 bp)
Lp. plantarum L125	+	+	+	+
Lp. pentosus L33	-	-	-	+
Lc. paracasei SP5	-	-	-	+
Lc. rhamnosus GG	-	-	-	+
Lc. casei ATCC 393	-	-	-	+
Lp. plantarum E4	-	-	-	+
Lp. plantarum E71	-	-	-	+
Lp. plantarum E73	-	-	-	+
Lp. plantarum B282	-	-	-	+



Yoghurt samples

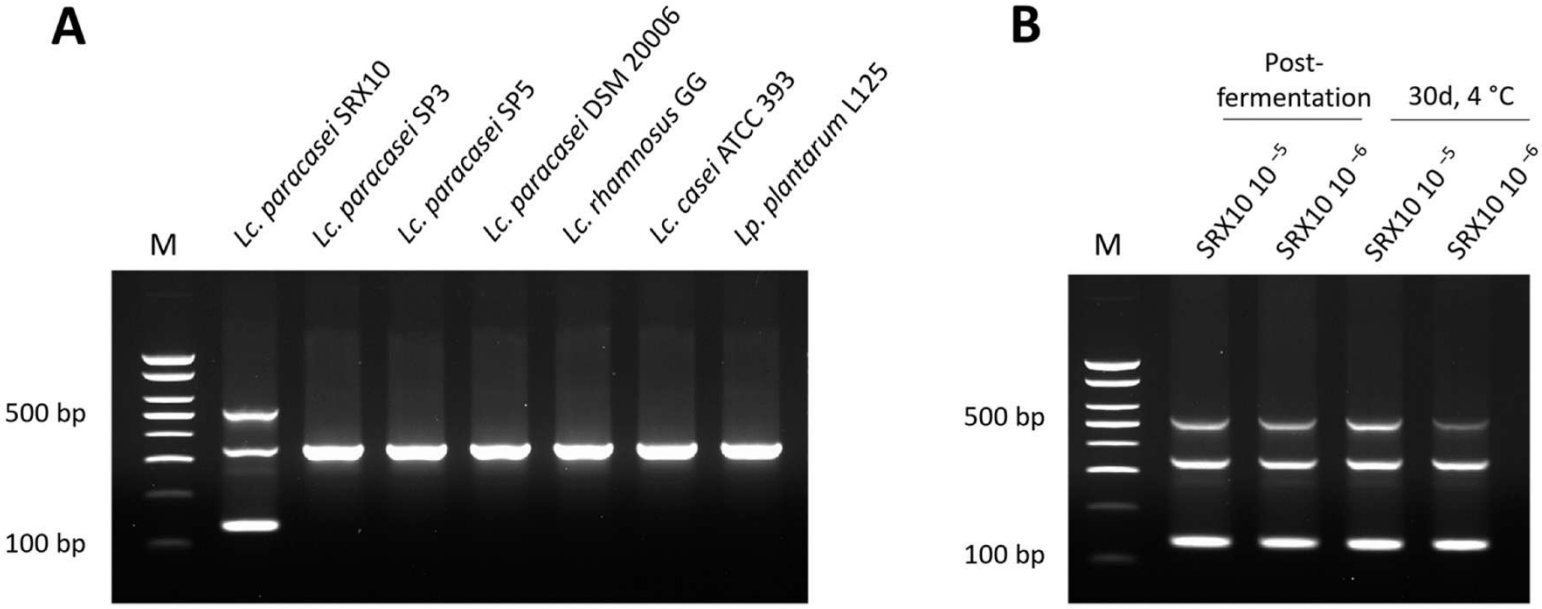




Dissecting the Genetic Basis of the Technological, Functional, and Safety Characteristics of *Lacticaseibacillus paracasei* SRX10

by Christina S. Kamarinou [1,2,†], Despoina E. Kiousi [1,†], Panagiotis Repanas [1], Anthoula A. Argyri [2], Nikos G. Chorianopoulos [3] and Alex Galanis [1,*]

Microorganisms **2024**, *12*(1), 93; https://doi.org/10.3390/microorganisms12010093



Metagenomic Insight into Cecal Microbiota Shifts in Broiler Chicks Following *Eimeria* spp. Vaccination

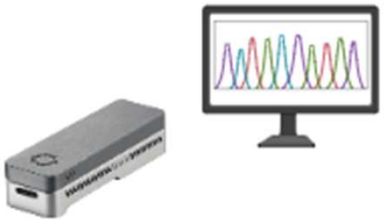
by **Dimitrios Marinos Karadedos** [1,†], **Tilemachos Mantzios** [1,2,†], **Despoina Eugenia Kiousi** [1], **Margaritis Tsifintaris** [1], **Ilias Giannenas** [3], **Panagiotis Sakkas** [3], **Georgios A. Papadopoulos** [4], **Gunther Antonissen** [5], **Aglaia Pappa** [1], **Alex Galanis** [1] and **Vasilios Tsiouris** [2,*]

Microorganisms **2025**, *13*(7), 1470; **https://doi.org/10.3390/microorganisms13071470**

Metagenomic analysis

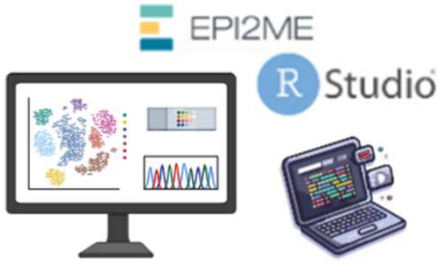
DNA isolation

MagCore® Gut Microbiome DNA Kit (Code: 504)

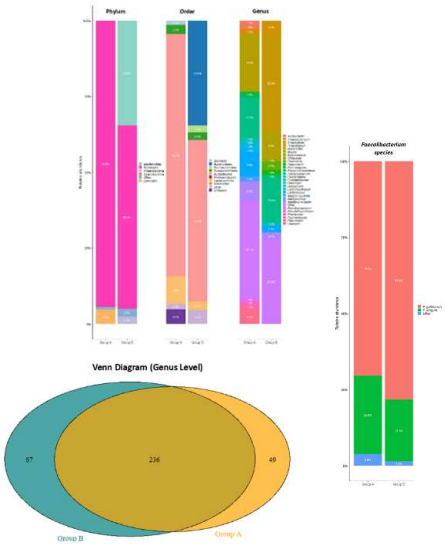


16S rDNA amplicon seq

MinKNOW
Oxford Nanopore

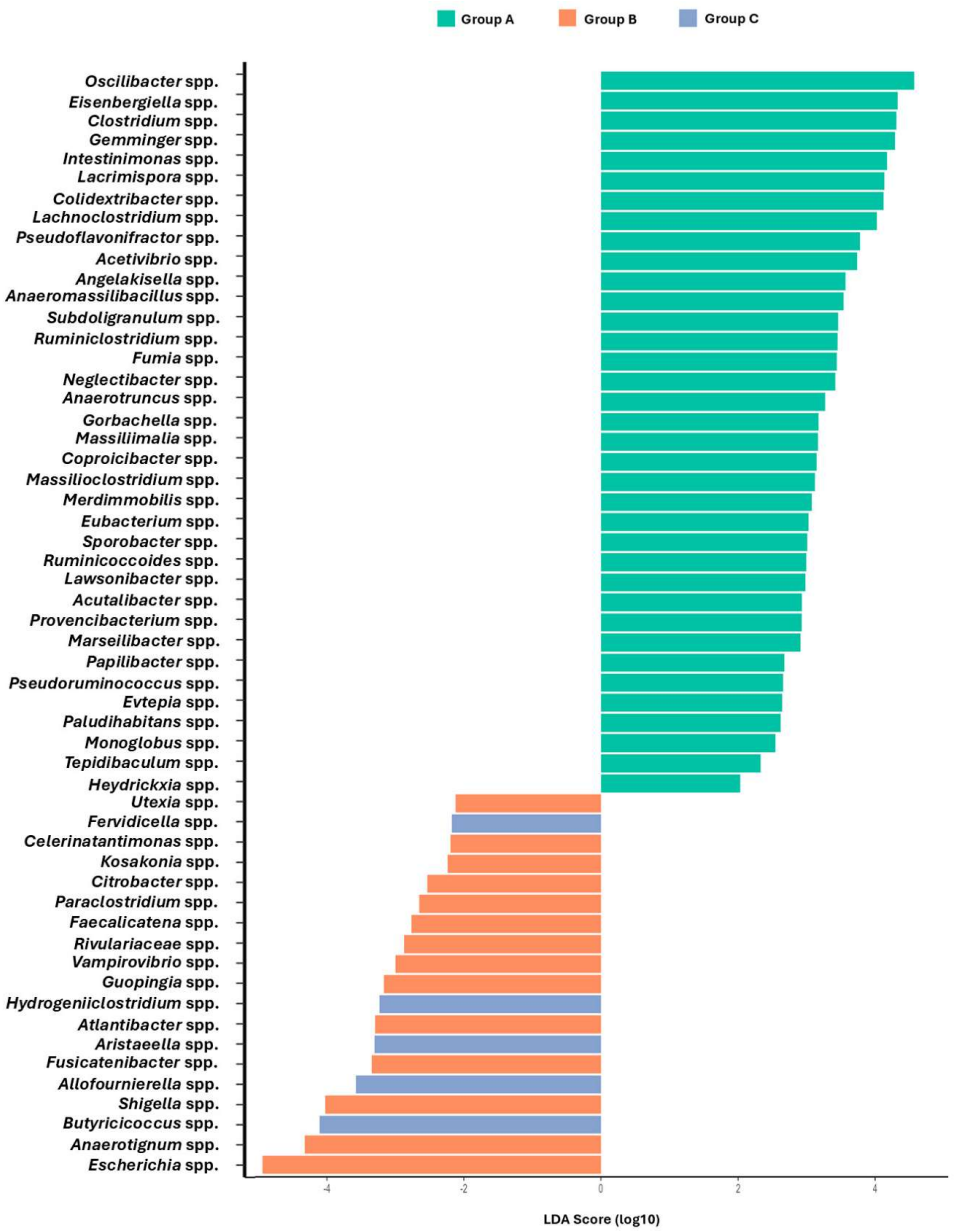


Bioinformatic Analysis

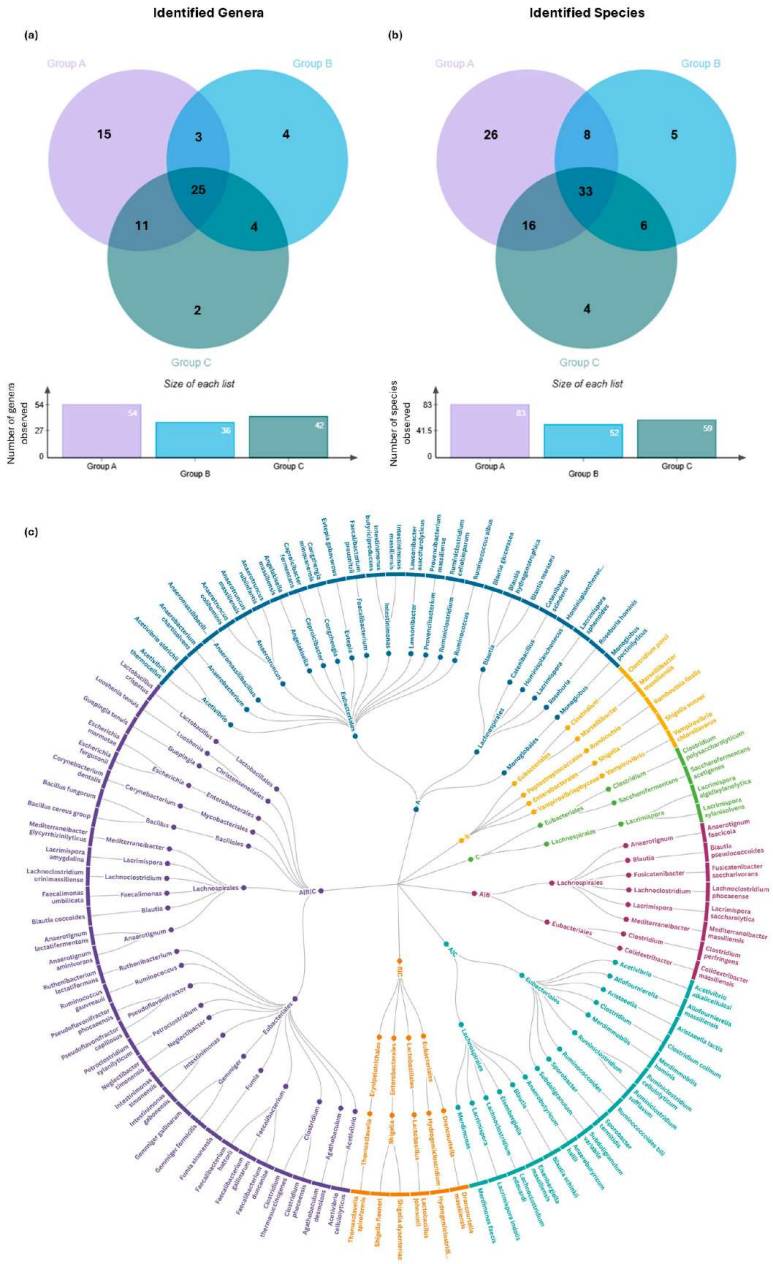


Taxonomic composition of cecal microbiota across experimental groups. Stacked bar plots represent the relative abundance (%) of bacterial taxa detected in cecal samples from group A, group B, and group C at four taxonomic levels; (**a**) phylum, (**b**) order, (**c**) genus, and (**d**) species. Only taxa with a relative abundance ≥ 1% in at least one group are shown individually; taxa below this threshold were grouped into the category "Other". Taxonomic classification was based on Operational Taxonomic Unit (OTU) clustering and relative abundances were calculated as percentages of the total sequences per sample.

Differentially abundant bacterial genera among groups based on Linear discriminant analysis effect size (LEfSe). LEfSe was used to identify genera with significantly different relative abundances among group A (control), group B (challenged with Eimeria spp.), and group C (vaccinated and challenged with Eimeria spp.). Only genera with an adjusted $p < 0.05$ and LDA score ($\log_{10}$) above the significant threshold ($\log_{10} > 2$) are shown. Genera enriched in group A are indicated by positive LDA scores (green), while genera enriched in group B and group C are shown with negative LDA scores in orange and blue, respectively.



Comparative analysis of core microbiota composition across experimental groups. (**a,b**) Venn diagrams display the number of shared and unique bacterial taxa among group A (control), group B (challenged with Eimeria spp.), and group C (vaccinated and challenged with Eimeria spp.) at the genus (**a**) and species (**b**) level. Taxa were included only if they were detected in at least three replicates per group, representing the core microbiota per each condition. Bar plots below each Venn diagram indicate the total number of genera and species identified in each group, respectively. (**c**) Circular cladogram representing the taxonomic relationships and distribution of genus-level bacterial taxa identified across the groups. From the outer to the inner rings, the plot shows the following: species (outer ring), genera (intermediate ring), and phyla (central nodes). Each branch of the cladogram is color-coded according to group-specific or shared taxonomic presence; blue for taxa shared between group A, yellow for group B, green for group C, plum for taxa shared between groups A and B, turquoise for groups A and C, orange for groups B and C, and purple for genera shared among all three groups. The cladogram integrated taxonomic hierarchy with group distribution to highlight both phylogenetic relationships and taxa overlap across groups.



Predictive functional analysis of metabolic pathways differently enriched among experimental groups. (**a**) Relative abundance of key metabolic pathways in the control group (group A, red) and the challenged group (group B, blue), with the corresponding log2 fold change between groups displayed on the right. Pathways enriched in group A have positive log2 fold change values, whereas those enriched in group B have negative values. (**b**) Relative abundance of statistically significant metabolic pathways in the control group (group A, red) and the vaccinated and challenged group (group C, blue), with the corresponding log2 fold change displayed on the right. Pathways enriched in group A have positive values, while those enriched in group C have negative values. Statistical significance (adjusted p-value) for each pathway was calculated using the DESeq2 algorithm, embedded in ggpicrust2 R package, and is shown adjacent to the log2 fold change bars. Relative abundance of pathways with adjusted $p < 0.05$ were considered statistically significant.