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Strain-specific qPCR assay to track the vaginal colonization of the probiotic *Lacticaseibacillus rhamnosus* TOM 22.8 strain orally administered

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Abstract

Background Over the past decade, research on the female reproductive tract microbiota has grown significantly and the use of probiotics has gained growing interest in restoring vaginal microbial balance. In this context, probiotic lactobacilli, topically or orally administered, emerged as a valuable alternative or a complementary approach to conventional antimicrobial therapies. Although topical application allows for a fast and direct action on the altered vaginal ecosystem, the oral intake provides long-term benefits due to the passage through the gastrointestinal tract.

Methods In the present study, in silico analyses were conducted to design a specific primer pair, which was validated by qPCR analysis and used to detect the orally administered *Lacticaseibacillus rhamnosus* TOM 22.8 probiotic strain in vaginal swabs of women with vaginal dysbiosis.

Results Data showed that qPCR confirmed the specificity of the strain-specific primer pair designed, and no amplification results were seen from 30 *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Lacticaseibacillus paracasei* strains used for comparison. In addition, the qPCR methodology allowed the detection of the *Lacticaseibacillus rhamnosus* TOM 22.8 probiotic strain in vaginal swab samples, with cell densities ranging from 5.24 log CFU/mL to 6.65 log CFU/mL.

Conclusion The approach used in the present study revealed the ability of the *Lacticaseibacillus rhamnosus* TOM 22.8 probiotic strain to survive during the GI tract passage and translocate into the vaginal ecosystem.

Keywords Woman health, Gut-vagina axis, Real-time PCR, Designed primer pair, Oral intake

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Introduction

Over the past decade, investigations on the female reproductive tract (FRT) microbiota have increased exponentially [1]. Probiotic administration in the gynaecological field is steadily advancing due to the growing interest in the treatment of diseases affecting the vaginal ecosystem. It is already well established that the probiotic lactobacilli-based approach is being extensively studied as an alternative to the conventional antimicrobial treatment [2]. Scientific evidence has demonstrated the beneficial effects of the use of probiotic lactobacilli, both topically or orally administered [3, 4]. While the topical administration of probiotics could exert a direct and quick action towards an altered vaginal microbiota, allowing a fast restoration of the eubiosis condition [5], the oral administration was well demonstrated as a long-term efficacy treatment, due to the passage through the gastrointestinal tract (GIT) [6]. In addition, recent studies suggested that oral administration of lactobacilli and bifidobacteria would colonize both the intestinal and vaginal mucosal surfaces [7]. Once established in the gut, they can positively modulate the intestinal ecosystem [8], enhance immune responses, and suppress the translocation of pathogens [9, 10].

Hence, the effectiveness of *Lactobacillus* as prophylaxis has been especially well-founded in long-term administration, through the production of surfactant molecules (peptidoglycan, lipoteichoic acid, exopolysaccharides, etc.), which antagonize pathogenic microbes, and the products of their glucose metabolism (lactic acid) contributing to an acidic (low pH) environment in the vagina, where they exert antimicrobial, antiviral, and immunomodulatory effects [11].

Although the vagina-gut axis is characterized by distal interactions, the rectum is relatively close to the vagina, it may be a site of exchange of some transient microbial species of the gut-vagina axis [1]. In this regard, the translocation phenomenon was recently revealed by the presence of the most prevalent vaginal *Lactobacillus* species (*Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, and *Lactobacillus gasseri*) to colonize the rectum. Rectal colonization with these microorganisms contributes to the maintenance of the health-associated vaginal microbiota, allowing a lower prevalence of bacterial vaginosis (BV) [12]. Several studies have shown that treatment with probiotics reduces recurrences in patients with bacterial vaginosis, enabling the rebalancing of the vaginal ecosystem [13], and furthermore, promoting both intestinal and vaginal ecosystem colonization, opening a new perspective for probiotic therapy in order to treat uro-genital infections and reduce disease recurrence [7].

The efficacy of a probiotic treatment is not only supported by symptomatic improvement, but is often complemented by microbiological investigation, which allows the identification and quantification of the administered probiotic. In recent years, microbial monitoring techniques have been revolutionised and replaced by faster and more accurate molecular methods. The combination of DNA fingerprinting and quantitative real-time PCR (RT-qPCR) provides a powerful tool to identify and quantify bacteria at the strain level, which has made this method popular for monitoring viable cells, viable but non-culturable (VBNC), or cell debris [14]. In particular, the design of strain-specific primers, projected from unique sequences or genes, allows for much more accurate results regarding the presence of the strain to be monitored [15]. Data obtained from studies conducted by Pino and coworkers [4] and Vaccalluzzo and coworkers [16], demonstrated the safety (e.g. absence of DNase, gelatinase, mucin-degrading enzymes, haemolytic activity, bile salt hydrolases, and antibiotics resistance) and functional properties (e.g. resistance to gastrointestinal passage, ability to adhere to Caco-2 and VK2/E6E7 epithelial cells, antioxidant, anti-inflammatory, and antimicrobial activities) of the *Lacticaseibacillus rhamnosus* TOM 22.8 (DSM 33500) strain as well as its effect in restoring and maintaining a balanced vaginal ecosystem [4, 16]. Based on this, we hypothesized a translocation from the gastrointestinal tract to the vaginal environment. Therefore, to prove this hypothesis, we developed a strain-specific quantitative PCR (qPCR) assay to detect the TOM 22.8 probiotic strain in vaginal swab samples, collected before and after the oral administration.

Materials and methods

De-novo genome assembly and annotation of the *Lacticaseibacillus rhamnosus* TOM 22.8 strain

The genome of the *Lacticaseibacillus rhamnosus* TOM 22.8 strain was fully sequenced through an Illumina MiSeq Sequencing System (unpublished data). Raw paired-end reads (FASTQ files, R1 and R2) were quality-checked with FastQC (0.11.9) to confirm high-quality data. The FASTQ files were used for *de novo* assembly of the draft genome of the *Lacticaseibacillus rhamnosus* TOM 22.8 strain with the Shovill (1.1.0) pipeline (https://github.com/tseemann/shovill), implementing SPAdes (v.3.15.4) genome assembler [17] using the MEGAnnotator pipeline [18] to perform *de novo* assemblies with pre- and post-assembly optimization steps. The overall quality of the final draft genome assembly was assessed with QUAST (v.5.1.0rc1) [19] and the completeness of the draft genome of the TOM 22.8 strain was evaluated with BUSCO (v.5.2.2), using the lineage dataset lactobacillales_odb10. The genome of the TOM 22.8 strain was annotated with Prokka (v.1.14.6) [20] using a customized genus-specific database created from all complete *Lactobacillus* and *Lacticaseibacillus* genomes in NCBI Assembly at the time of the study.

The phylogenetic relationships between the *Lacticaseibacillus rhamnosus* TOM 22.8 strain and 202 strains belonging to the same species were assessed by phylogenomic analysis with the UBCG pipeline [21] (92 core genes). The available RefSeq genome assemblies of 202 *Lacticaseibacillus rhamnosus* strains, downloaded from NCBI, were used to infer the phylogenetic relationships of the TOM 22.8 strain, using a bacterial core gene set that was defined by an up-to-date bacterial genome database. The phylogenetic relationships of clades were further investigated by performing an extensive core genome Multi-Locus Sequence Analysis (cgMLSA) of 2132 loci on the TOM 22.8 strain and co-clustering *Lacticaseibacillus rhamnosus* strains' genomes, using the GET_HOMOLOGUES pipeline [22]. The identified clusters of orthologous gene sequences were aligned (MAFFT v7.453), trimmed, and concatenated in a supermatrix. The core genome supermatrix was used to reconstruct a Neighbor-Joined tree with MEGA11, using the Tamura-Nei distance algorithm. The sequence and structural similarity of the genomes of the selected strains were evaluated using MUMmer4 dnadiff [23], a wrapper for MUMmer4's nucmer and analysis utilities able to provide detailed information on the differences between genomes and also to obtain a high-level report file that quantifies the differences between the two inputs.

Design and validation of strain-specific primers

Based on the unique region identified, the primer pair TOM_F (AATGTCTGCGAGTTCTGCCTTT) and TOM_R (ACTGCTGTGCGTCGTA) were designed and validated, by both endpoint PCR and quantitative PCR (qPCR), using the DNA extracted from pure cultures of the *Lacticaseibacillus rhamnosus* GG (ATCC 53103) and of 30 *Lacticaseibacillus rhamnosus* strains, 5 *Lacticaseibacillus casei* strains, and 5 *Lacticaseibacillus paracasei* strains from the ProBioEtna culture collection. These lactobacilli strains were previously isolated from different human and food ecosystems and were identified at the species level through species-specific PCR. The DNA isolated from a pure culture of the *Lacticaseibacillus rhamnosus* TOM 22.8 strain was used as a positive control. The DNA was isolated following the method described by Vaccalluzzo et al. [24]. The endpoint PCR was performed in a total volume of 25 µL containing 12.5 µL of 2X YourTaq™ PCR Master Mix (BiotechRabbit, Berlin, Germany), 10 µM of each primer, 1 µL of the DNA template, and 9.5 µL of Dnase/Rnase free water. The PCR amplification cycle was as follows: 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s followed by a final extension at 72 °C for 5 min.

For primers validation thought qPCR, a standard curve was generated using the gDNA isolated from the TOM 22.8 strain. In detail, the detection threshold of the *Lacticaseibacillus rhamnosus* TOM 22.8 strain was evaluated by performing 10-fold serial dilutions covering a range of cell densities from 10^8 to 10^3 genome copies/µL. This allowed the correlation of Ct values with genome copy numbers, and the quantification of vaginal samples was expressed as $\log_{10}$ DNA copies/µL. To monitor sample quality and exclude the presence of PCR inhibitors, an internal amplification control, targeting the bacterial 16 S rRNA gene, was conducted according to the protocol proposed by Agolino and collaborators [25]. The qPCR mix contained 1 µL of the DNA template, 10 µL of QuantiFast SYBR Green Mastermix (Qiagen, Milan, Italy), 10 µM of each forward and reverse primer, and 7 µL of Dnase/Rnase free water. Cycling conditions were 95 °C for 15 min followed by 45 cycles of 95 °C for 30 s, 62 °C for 30 s and 62 °C for 30 s. The slope of the regression curve between the DNA concentrations log values and the average Ct values was used to calculate the primers efficiency using the equation: E = 0.5 (10(−1/slope)) x100. A melting curve analysis (60 °C to 95 °C) was performed to ensure specificity in the amplification. The qPCR reactions were performed in triplicate using a Rotor Gene Q instrument (Qiagen, Milan, Italy).

DNA isolation from vaginal swabs and detection of the TOM 22.8 strain by strain-specific qPCR

Vaginal swabs used in the present study were collected during a single-centre, randomized, controlled clinical trial, previously described by Vaccalluzzo and co-workers [16]. In detail, participants allocated to the active group received one daily oral capsule for 10 consecutive days, each containing 1×10^{10} CFU (10 billion dose) of the *Lacticaseibacillus rhamnosus* TOM 22.8 (DSM 33500). The swabs were collected from healthy volunteers with vaginal signs and symptoms of vaginal dysbiosis before (baseline, T0) and after 10 days (T1) of treatment with the *Lacticaseibacillus rhamnosus* TOM 22.8 strain. In addition, vaginal swabs from untreated patients (placebo group) were collected. The volunteers were informed regarding the procedures they underwent and signed an informed consent form for data collection, which was approved by the Institutional Review Board (IRB) of the Policlinico hospital of the University of Catania (registration number 157/2019/PO).

Total genomic DNA was extracted from 30 swab samples from the active group and 10 swabs from the placebo group, collected at both T0 and T1 sampling times. Strain-specific qPCR was performed in triplicate using a Rotor Gene Q instrument (Qiagen, Milan, Italy).

Statistical analysis

To detect differences in the *L. rhamnosus* TOM 22.8 detection levels among the vaginal swab samples, data related to qPCR, expressed as $\log_{10}$ copies of DNA/µL,

were subjected to one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$. The statistical analysis was performed using the Statistica software (version 10.0 for Windows, TIBCO Software, Palo Alto, CA, USA).

Results

Design of strain-specific primer pair

The genome size obtained was 2.964.508 bp. The FastQC (0.11.9) basic sequence statistics are reported in Table S1. Overall, a total of 278,346 reads were obtained, and no sequences were flagged as poor quality. Sequence length ranged from 35 to 250 bp and the observed GC content was 46%. Were detected. Results of the overall quality of the final draft genome assembly, assessed with QUAST (v.5.1.0rc1), are displayed in Table S2. The de novo assembly generated 11 contigs, with a total length of 2,959,300 bp. Six contigs exceed 10,000 bp and the largest contig was approximately 1,005,919 bp. The GC percentage was 46.70%, N50 and N90 values were 784,517 bp and 411,851 bp, respectively. High contiguity, with an L50 of 2 and an L90 of 4, was observed (Table S2). A summary of the draft genome completeness of the TOM 22.8 strain, assessed with BUSCO, is reported €n Table S3. The BUSCO analysis showed high completeness, with 99.5% of the 402 conserved orthologs identified as complete €, of which 99.0% were single-copy (S) and only 0.5% were duplicated (D). Fragmented orthologs represented only the 0.5% (F) and no missing BUSCOs (M = 0.0%) were observed (Table S3). Based on the draft genome annotation with Prokka (v.1.14.6) and according to the *Lactobacillus* and *Lacticaseibacillus* genus-specific database the gene prediction yielded a total of 2748 predicted coding sequences and 569 identified hypothetical proteins. Based on the results obtained using the UBCG pipeline, 2132 clusters of orthologous gene sequences were identified among 9 genomes, aligned (MAFFT v7.453), trimmed, and concatenated in a supermatrix of 1,958,844 nucleotide positions x 9 OTUs and the number of differences (single nucleotide polymorphism) in pairwise comparisons is reported. Detailed pairwise comparisons are provided in Table S4, supporting the genetic distinctiveness of the TOM 22.8 strain. In the frame of evaluating the genetic similarities between the *Lacticaseibacillus rhamnosus* strains' genomes, MUMmer4 dnadiff provides complementary data to the phylogenomic information, with the identification and description of differential regions in the compared genomes, such as unaligned unique contigs and major structural differences in the aligned regions. The differential primers were designed on a variable region coding for a putative phage protein: the homologous region in *Lacticaseibacillus rhamnosus* GG (NCBI Acc. No.: CP031290 is annotated as locus DU507_15115) named "phage major capside protein". The variable region identified was submitted to the BankIt, under the GenBank accession number PQ857571. The designed primer pair was TOM_F (AATGTCTGCGAG TTCTGCCTTT) and TOM_R (ACTGCTGTGCGTC GTA), and the predicted amplicon size was 176 bp. The details of the primer sequences are provided in Table 1.

Validation of strain-specific primers

The specificity of the designed primer pairs was validated by the method described above. The end-point PCR analysis, targeting the "main phage capsid protein" for the detection of the *Lacticaseibacillus rhamnosus* TOM 22.8 strain, proved the specificity of the designed primers. In fact, as displayed in Figure S1, no amplification was detected for the closely related strains belonging to the species *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus casei*. According to the obtained results, the qPCR analysis, with a coefficient of determination (R^2) of 97.0% and an amplification efficiency (E) of 104%, confirmed the specificity of the designed strain-specific primers. The validation of strain-specific primers confirmed the robustness of the qPCR test. Amplification curves showed efficient and reproducible detection in serial dilutions, while melt curve analysis revealed the presence of two peaks, one corresponding to the reference strain and one corresponding to all strains used for validation (Fig. 1, panels A-C). No amplification results for the 30 strains of *Lacticaseibacillus rhamnosus*, 5 strains of *Lacticaseibacillus casei*, and 5 strains of *Lacticaseibacillus paracasei* tested in comparison, with the exception of *Lacticaseibacillus rhamnosus* TOM 22.8, were revealed.

Strain-specific qPCR of vaginal swab samples

Based on the detection threshold data, a coefficient of determination (R^2) of 99.2% was obtained. According to the slope value obtained (E = 10^(1-/slope), the amplification efficiency (E) was 106%. Based on the standard curve, the detection threshold varied between 8.0 $\log_{10}$ DNA copies/µL and 3.04 $\log_{10}$ DNA copies/µL with cycle threshold (Ct) values of 6.73 and 23.23, respectively. The cycle threshold and quantitative data, expressed as $\log_{10}$ DNA copies/µL and standard deviation, are shown in Table S5 and Fig. 2. Although statistically significant

Table 1 References of the strain-specific primer pair

Primer	Sequence	Position (5′–3′)	Length	Secondary structure	Primer dimers	Tm (°C)	GC (%)
TOM-F	AATGTCTGCGAGTTCTGCCTTT	741–762	22	None	No	66.10	45.45
TOM-R	ACTGCTGGTGCGGTCGTA	919 – 900	18	None	No	65.80	61.11

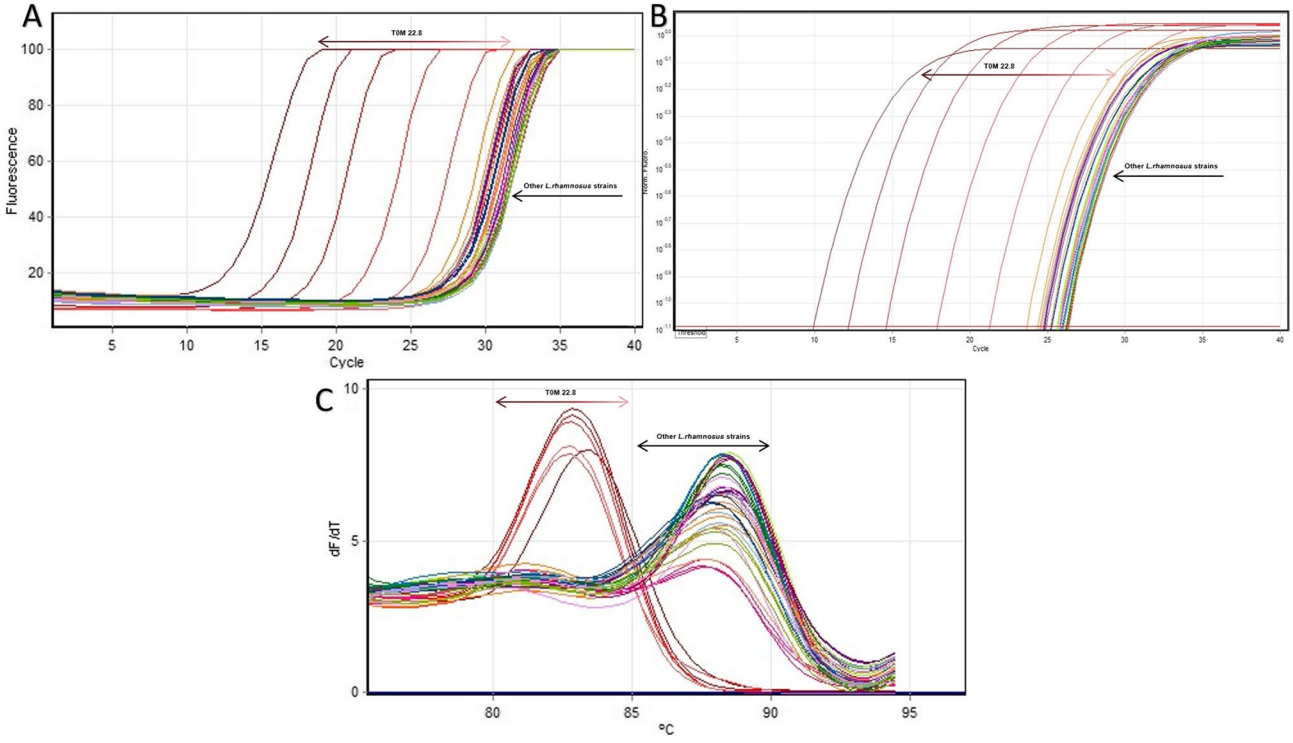

Fig. 1 Validation by qPCR with primers specific for the *Lacticaseibacillus rhamnosus* TOM 22.8 strain. Analysis of amplification and melting curves of the reference strain (serially diluted, standard) compared to other strains belonging to the *L. rhamnosus* species. (**A**) Raw cycle data; (**B**) Cycle quantification data; (**C**) Melting data

differences were detected among samples, the *Lacticaseibacillus rhamnosus* TOM 22.8 strain was revealed in all the vaginal swabs collected at T1 sampling time from the active group, with cell densities ranging from 5.24 $\log_{10}$ DNA copies/µL to 6.65 $\log_{10}$ DNA copies/µL and Ct values of 20.82 and 11.24, respectively, and a mean value of 6.26 ± 0.43 $\log_{10}$ DNA copies/µL. Figure 3 (panels A-D) shows amplification curves with a steady, exponential increase in fluorescence, confirming the detection of the target strain in the vaginal swab samples. Furthermore, the melting curve confirmed the presence of a single, clear peak at the expected melting temperature, indicating the specificity of the amplification and ruling out the formation of primer dimers or non-specific products.

No amplification was obtained from the gDNA isolated from vaginal swabs collected at baseline (T0) and from untreated patients (placebo).

Discussion

Currently, the phenomenon of microbial translocation is one of the most extensively discussed topics in the scientific community, especially with regard to the study of direct and indirect interactions between organs. Focused on the FRT ecosystem, the interactions of the "gut-vagina axis" have been increasingly supported by clinical studies, proving that orally administered probiotics enable to restore the homeostasis of the vaginal microbiota. It was demonstrated that lactobacilli, oral administered, can colonise both rectal and vaginal niches in women over a period of several weeks [26]. Furthermore, randomised studies using combinations of oral probiotics (*Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus*, *Limosilactobaciullus reuteri*) have reported a significant enrichment of vaginal *Lactobacillus* spp. and a reduction in dysbiosis-associated bacteria, supporting the biological plausibility of an intestinal influence on the vaginal microbiota even in the absence of evidence of direct translocation [27–29]. Several hypotheses have been put forward for a rectal migration, as the rectum can act as a reservoir for vaginal strains belonging to the *Lactobacillus* spp. genus and facilitate microbial exchange between adjacent sites [30]. Other studies have supported a systemic mucosal interaction in which the intestinal microbial composition can modulate distant mucosal surfaces, potentially through immunometabolic or hormonal pathways, influencing vaginal health beyond localised colonisation [1, 12, 31–33]. These pathways are not mutually exclusive and future studies, combining longitudinal faecal, rectal, vaginal, and systemic sampling, will be necessary to clarify the dynamics of this process. In this context, Strus and co-workers [26] assessed the degree and persistence of colonization by a blend of lactobacilli strains (*Lactobacillus fermentum* 57 A, *Lactobacillus plantarum* 57B, and *Lactobacillus gasseri* 57

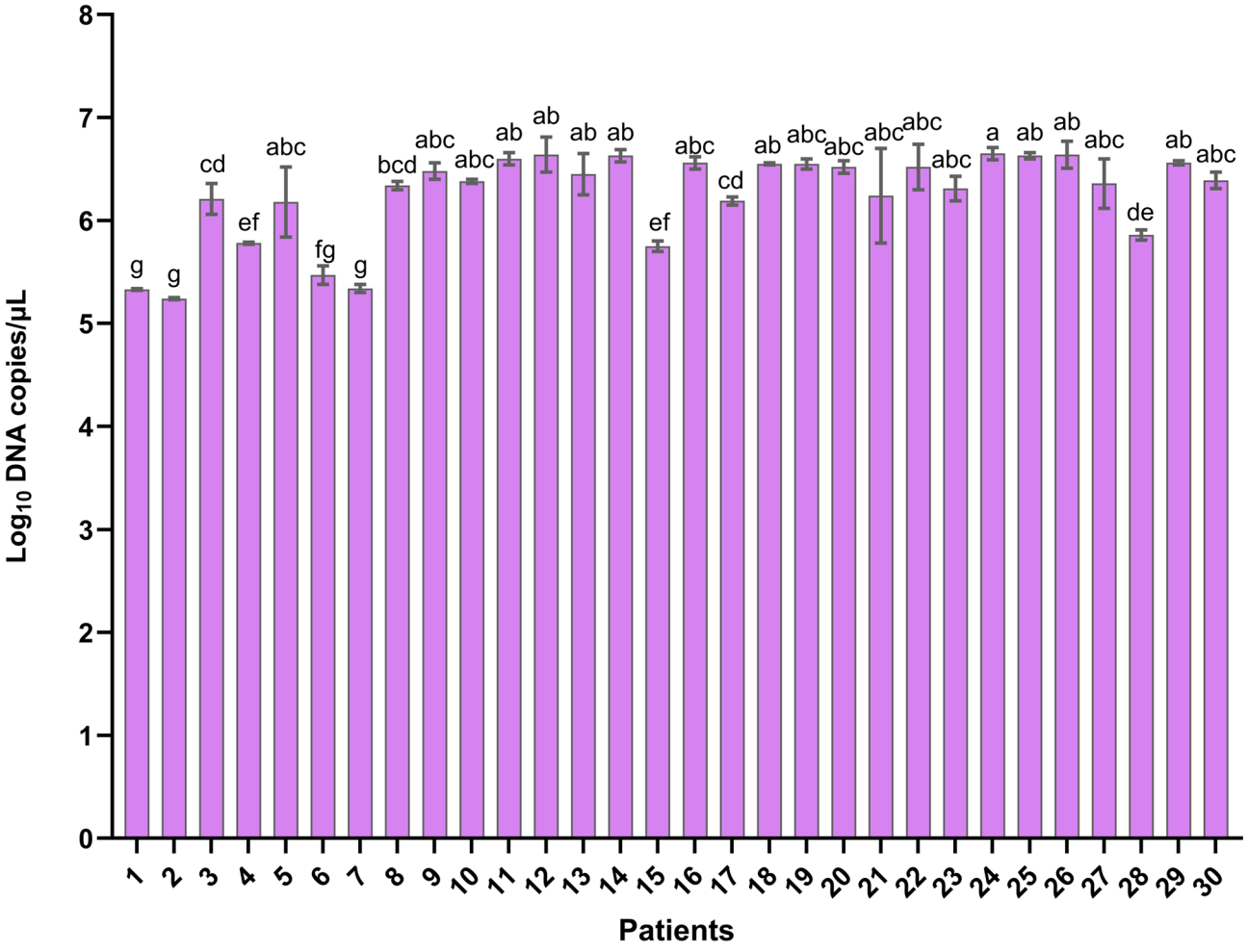

Fig. 2 The bar graph displays the quantification values of *Lacticaseibacillus rhamnosus* TOM 22.8 in vaginal swab samples collected from 30 patients after 10 days of oral administration (T1). The data are expressed as log10 DNA copies/µL ± standard deviations. a-g, different superscript letters indicate significant differences at $p < 0.05$

C), orally administered. By analysing vaginal and rectal samples through molecular typing methods, the authors demonstrated that the ingested strains were able to reach and colonize both sites. Similarly, Lin et al. [34] investigated the therapeutic effect of a Lactobacillus formula, namely VGA-1 and VGA-2, in BV patients, as well as their ability to reach the vagina. Along with revealing a significant improvement in vaginal discharge odor/color and itching, the authors, by studying the RAPD profiles of lactobacilli isolated from swab samples, confirmed the presence of the VGA-1 formula in the vagina.

The present work sheds light on how the use of a sophisticated methodology, based on the use of validated primers exclusively designed for the target strain, is capable of qualitatively and quantitatively detecting the orally administered probiotic. Furthermore, it corroborated not only its ability to survive during passage through the gastrointestinal tract but also the strain adhesion on the vaginal mucus. In this regard, a comparative study, regarding the delivery method, conducted by Mändar and coworkers [6], showed that whereas vaginally administration of *Lactobacillus crispatus* was able to reach the point of action without delay, oral administration, however, equally enabled the reduction of BV-related bacteria, making it a more suitable and convenient mode of administration for the patient. Although oral administration was validated for its efficacy in the treatment of vaginal dysbiosis, there are few studies that use methods capable of verifying the actual presence and survival of the administered probiotic and thus strictly tracing it to the positive effects conferred on the vaginal tract. Indeed, the use of sophisticated molecular methods, such as real-time PCR, allows the identification of target probiotics using species- or strain-specific primers/probes with high sensitivity, speed, and accuracy. Moreover, they allow not only a qualitative investigation of the presence/absence of the target probiotic, but also its quantitative detection [35]. In addition, to validate the efficacy of an orally

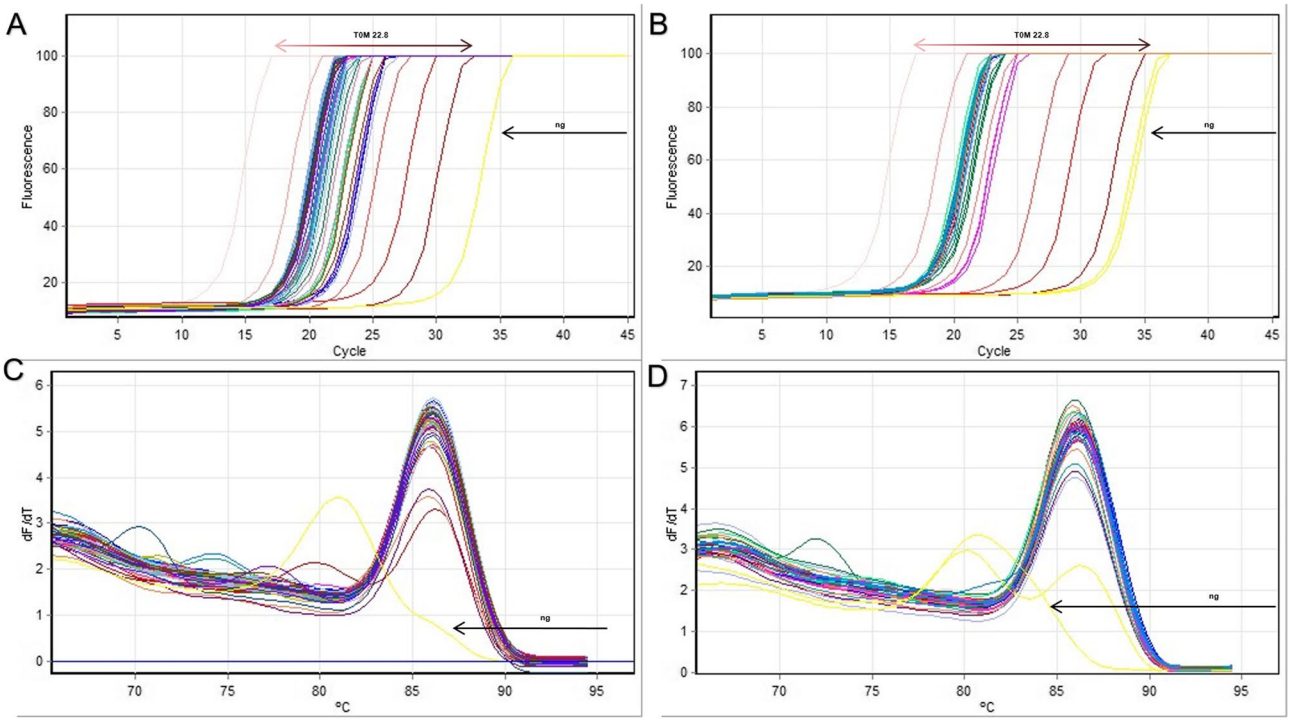

Fig. 3 Representative amplification curves (**A**-**B**) and melting curves (**C**-**D**) obtained from vaginal swab samples of enrolled patients analysed with strain-specific primers targeting the *Lacticaseibacillus rhamnosus* TOM 22.8 strain. ng: negative control

administered probiotic in a complex ecosystem, such as the human body, typing methods for identifying target strains appear to be the most suitable choice. However, they require time and specialised expertise or advanced hardware and software that can identify probiotic strains from all other strains belonging to the same species. Identifying a target strain requires conducting a gene family analysis, so as to identify strain-specific gene families, and finally identifying strain-specific sequences [15]. In the present study, a core genome multi-locus sequence analysis was conducted by correlating several genomic profiles of *Lacticaseibacillus rhamnosus* strains with the *Lacticaseibacillus rhamnosus* TOM 22.8 consensus genome. The phylogenetic analysis allowed identification of a unique region for the target strain, and the designed primer pairs were validated for the selected strain detection. In a previous study [16], we confirmed the ability of *Lacticaseibacillus rhamnosus* TOM 22.8 strain to treat vaginal dysbiosis and to increase the abundance of lactobacilli in the complex vaginal ecosystem. Specifically, using a qPCR genus-specific approach, we noticed, at the end of treatment (T1), an increase in the lactobacilli population of about 4 log units (8.30 log units CFU/mL) compared with the beginning of treatment, which was also validated by the culture-dependent method.

This quantitative methodology was also confirmed in a pilot study conducted by De Alberti and collaborators [36] and further Russo and coworkers [37], in which the oral intake of Respecta® capsule, a formulation that combined *Lactobacillus acidophilus* and *Lacticaseibacillus rhamnosus* strains with lactoferrin complex, was detected by real-time PCR using a species-specific primer pair. The treatment allowed an increase in the species administered in the vagina, detecting persistence of the probiotic formulation even after 7 days after cessation of dosing, compared with placebo samples.

Specifically, the estimated amounts at baseline and at the end of treatment were about 2 $\log_{10}$ genomes per ng DNA and 3 $\log_{10}$ genomes per ng DNA for *Lacticaseibacillus rhamnosus* and *L. acidophilus*, respectively [37]. However, both studies reported visual confirmation of the quality of the dissociation curve, but without comparison with a standard. Therefore, it is not possible to define with certainty whether the increase in the lactobacilli population is favoured by the administration of the probiotic blend or depends on the presence of commensal microorganisms.

An additional study was conducted by Mezzasalma and coworkers [7], where the use of qPCR with species-specific primer pairs allowed the persistence of the orally administered probiotic complex to be monitored in the vaginal microbiota of healthy pre-menopausal women from the start of treatment to the follow-up period. Although the primer pair used cannot specifically assess the presence of the individual orally administered strains, the methodology proved effective in

determining the abundance of these species compared to placebo samples. Focusing on strain level, in a randomized, placebo-controlled, triple blind study, orally administered strains of *L. acidophilus* La-14 and *Lacticaseibacillus rhamnosus* HN001 were detected using the qPCR method with strain-specific primer pairs [38]. However, the cell density of the strains was less than 5.29 or 5.11 log units, while in the present study the high persistence of the administrated *Lacticaseibacillus rhamnosus* TOM 22.8 strain was revealed. In a study by Koirala and coworkers [39], a dual approach based on qPCR and 16 S rRNA gene sequencing, was applied to detect orally integrated *Lacticaseibacillus paracasei* LPCS01 strain. Vaginal strain-level colonization was detected at about 4–6 logs below the level of total bacteria present in a vaginal sample, down to about 5 Log10 cells/mL. Compared to previous clinical trials [7, 26], the present study used a strain-specific qPCR assay that targets a unique genomic region of *Lacticaseibacillus rhamnosus* TOM 22.8, allowing for precise monitoring of the administered strain, whereas previous studies used species-level primers, which are unable to distinguish among administered probiotics and endogenous lactobacilli. Furthermore, the colonisation levels detected in our study (5.24–6.65 log CFU/mL) were higher than those reported in the above-mentioned studies, which reported lower levels (2–3 log genome/ng DNA) or provided only relative abundances. Furthermore, our sampling strategy included baseline and treatment time point (10 days), allowing for a direct assessment of short-term colonisation dynamics. Finally, unlike the multi-strain formulation containing lactoferrin, evaluated by Russo [37] and Mezzasalma [7], we studied a single-strain probiotic, thus isolating the effects attributable specifically to this strain.

However, the study reported some limitations that must be taken into account, from the relatively small number of samples, which could limit the generality of the results, to the impossibility of assessing long-term colonisation, the persistence beyond the 10-day administration, and the effects and interactions with the overall vaginal and intestinal microbiota.

In addition, future research will include an integrated approach based on metagenomics including vaginal and faecal samples, clarifying the mechanisms of the gut-vagina microbial exchange.

Conclusion

In conclusion, in this study, the strain-specific qPCR assay allowed to track the *Lacticaseibacillus rhamnosus* TOM 22.8 after oral administration and its ability to colonize the vaginal ecosystem, providing direct evidence of translocation along the gut-vagina axis. These findings have important clinical implications, as they support the use of the oral administration of the TOM 22.8 strain as a safe and effective strategy to restore the balance of the vaginal ecosystem, potentially reducing the recurrence of dysbiotic conditions and offering a complementary option to conventional antimicrobial therapies. Furthermore, the validated molecular approach applied in this study represents a reliable tool for future clinical monitoring of probiotic colonization and persistence at the strain level.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04470-x.

Supplementary Material 1: Table S1. FastQC basic sequence statistics. Table S2. QUAST report of the TOM 22.8 strain genome assembly. All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs). Table S3. Short summary results of the completeness of the TOM 22.8 strain draft genome assessed with BUSCO. Table S4. Pairwise comparisons of the nucleotide sequences computed in MEGA 11. Table S5. Quantification of the ***Lacticaseibacillus rhamnosus*** TOM 22.8 in vaginal samples.

Supplementary Material 2: Figure S1. Validation by end-point PCR on electrophoresis gel of strain-specific primers.

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Authors' contributions
Conceptualization: A.P., C.L.R; Methodology: A.P., C.L.R; Investigation: A.V.; Formal analysis: A.V., G.B; Data curation: A.V., A.P., G.B.; Validation: A.P., C.L.R; Writing-original draft: A.V.; Writing-review and editing: A.P., C.C.; C.L.R; Supervision: C.C., C.L.R. All authors have approved the final version of the manuscript.

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Data availability
The variable region, coding for a putative phage protein, used to design the strain-specific primers was submitted to the BankIt (BankIt2909495 TOM22.8_contig00001_828599-827055), under the GenBank accession number PQ857571.

Declarations

Ethics approval and consent to participate
The studies involving humans were approved by the ethics committees of the Azienda Ospedaliero-Universitaria "Policlinico-Vittorio Emanuele" Catania, Catania 1, (registration number 157/2019/PO). The study was registered to clinicaltrial.gov (ID: NCT05817292, 04/04/2023). The study was conducted in accordance with the Declaration of Helsinki. All participants provided their written informed consent to participate in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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