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Substitution of Asp29 with Asn29 in the metallochaperone UreE of *Streptococcus thermophilus* DSM 20617^T increases the urease activity and anticipates urea hydrolysis during milk fermentation

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ABSTRACT

Urease operon is highly conserved within the species *Streptococcus thermophilus* and urease-negative strains are rare in nature. *S. thermophilus* MIMO1, isolated from commercial yogurt, was previously characterized as urease-positive Ni-dependent strain. Beside a mutation in *ureQ*, coding for a nickel ABC transporter permease, the strain MIMO1 showed a mutation in *ureE* gene which code for a metallochaperone involved in Ni delivery to the urease catalytic site. The single base mutation in *ureE* determined a substitution of Asp_{29} with Asn_{29} in the metallochaperone in a conserved protein region not involved in the catalytic activity. With the aim to investigate the role Asp_{29} *vs* Asn_{29} substitution in UreE on the urease activity of *S. thermophilus*, *ureE* gene of the reference strain DSM 20617^T ($ureE_{DSM20617}$) was replaced by *ureE* gene of strain MIMO1 ($ureE_{MIMO1}$) to obtain the recombinant ES3. In-gel detection of urease activity revealed that the substitution of Asp_{29} with Asn_{29} in UreE resulted in a higher stability of the enzyme complexes. Moreover, the recombinant ES3 showed higher level of urease activity compared to the wildtype without any detectable increase in the expression level of *ureC* gene, thus highlighting the role of UreE not only in Ni assembly but also on the level of urease activity. During the growth in milk, the recombinant ES3 showed an anticipated urease activity compared to the wildtype, and analogous milk fermentation performance. The overall data obtained by comparing urease-positive and urease-negative strains/mutants confirmed that urease activity strongly impacts on the milk fermentation process and specifically on the yield of the homolactic fermentation.

1. Introduction

Urease activity of *S. thermophilus* has been widely investigated in relation to its effect on milk fermentation processes (Della Scala et al., 2019; Yamauchi et al., 2019; Monnet et al., 2004; Mora et al., 2004; Pernoud et al., 2004; Yu et al., 2019) and to its role in cell physiology and metabolism (Arioli et al., 2010; Arioli et al., 2022). The intracellular urease (EC 3.5.1.5) of *S. thermophilus* catalyzes the hydrolysis of urea to yield ammonia and carbamate, which spontaneously decomposes to produce a second molecule of ammonia and carbonic acid. The release of ammonia in cytoplasm has several effects on *S. thermophilus* physiology. Ammonia release led to a transient increase of the intracellular pH thus modulating with the activity of all enzymes governing the metabolism (Arioli et al., 2010; Arioli et al., 2022). In addition, ammonia is a nitrogen source used by *S. thermophilus* to synthetize aspartate, glutamine, arginine, purine and pyrimidine (Arioli et al., 2007; Arioli et al., 2009a; Arioli et al., 2009b; Monnet et al., 2005). The excess of ammonia not used in catabolic reactions freely diffuses across the cell membrane thus alkalizing the environment surrounding urease-positive cells. This extracellular alkalization concomitantly to the development of the homolactic fermentation, resulted in a decrease of acidification which is considered a negative technological trait in dairy fermentation processes (Monnet et al., 2004; Mora et al., 2004; Pernoud et al., 2004). Apart from its physiological and technological relevance the biochemistry of *S. thermophilus* urease complex was never investigated. *S. thermophilus* urease is a complex enzyme Ni-dependent that results from the assembly of three subunits (Mora et al., 2004). Moreover, the activation of urease depends on a metallochaperone, an intracellular Ni-binding protein that protect the cell from the reactivity of free Ni ions while delivering them to a urease apoprotein. In all urease-positive bacteria, *S. thermophilus* included, the metallochaperone is identified in the UreE protein, which is characterized by a histidine-rich region at its carboxyl terminus that

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binds equivalents of Ni to be transferred to the urease apoprotein active site. Most of the studies on the assembly of an active urease, and specifically on the role of the metallochaperone UreE, have been carried out on urease of *Helicobacter pylori*, *Klebsiella aerogenes*, *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) (Ciurli et al., 2002; Hu et al., 2018; Lee et al., 1993; Merloni et al., 2014; Yang et al., 2015). For a comprehensive review of the function of UreE in the assembling of an active urease see Musiani et al. (2004) and Tsang and Wong (2022). Here we investigated the role of natural alleles of *ureE* gene of *S. thermophilus* urease activity. In previous study, we isolated from a commercial yogurt the urease-positive Ni-dependent *S. thermophilus* MIMO1 (Mora et al., 2002) which showed detectable urease activity only when cultivated in medium supplemented with $NiCl_2$ (Della Scala et al., 2019). Sequence comparison of urease gene cluster between strain MIMO1 and the reference DSM 20617^T revealed amino acid substitutions in UreE (Asp_{29} *vs* Asn_{29}) and UreQ (Asp_{270} *vs* Gly_{270}) (Della Scala et al., 2019). Whereas the mutation in *ureQ* gene could be involved in a defective Ni ions intake thus justifying its anomalous Ni-dependent urease activity, the mutation in *ureE* gene could have a role in Ni delivery to the urease active site. In this study the effect of Asn_{29} mutation in UreE on urease activity during milk fermentation was investigated by *ureE* gene replacement in the reference strain DSM 20617^T.

2. Materials and methods

2.1. Bacterial strains and growth condition

Wildtype *S. thermophilus* DSM 20617^T and its derivatives, A16, ES3, were cultivated at 37 °C in M17 broth (Difco Laboratories, Detroit, MI, United States) containing 2 % (w/v) lactose. Transformants ES3P and ES3PI were cultivated in M17 broth (2 % lactose) supplemented with 4 μg/ml and 2 μg/ml erythromycin respectively. ES3P was cultivated at 30 °C whereas ES3PI was cultivated at 42 °C. All strains and their description are listed in Table 1.

2.2. Replacement of $ureE_{DSM20617}$ with $ureE_{MIMO1}$ by allelic exchange in *S. thermophilus DSM 20617^T*

DNA manipulation of pG$^+$host9 vector and derivatives were carried out in *Escherichia coli* VE7108 (Mora et al., 2004). Plasmid isolation was performed using the Nucleospin plasmid Kit according to the manufacturer's (Machery-Nagel GmbH and Co., Düren, Germany) instructions. Electrotransformation of *S. thermophilus* DSM 20617^T was performed as described by Buckley et al. (1999). Vector pMI1 was obtained by cloning *ureE* gene fragment amplified from *S. thermophilus* MIMO1 into the dephosphorylated *Eco*RV site of pG + host9. Primers used for amplification of MIMO1 *ureE* fragment are the follow: UreE-EcoRV1f (GTAGATGCTCTTGTTAAGGATATCGATGT), UreE-EcoRVr (TTTCAAGGATATCTTGCAAAGACACATTAGC). Transformation of DSM 20617^T with pMI1 allowed the selection of erythromycin resistant transformants on M17 plates (2 % *w/v* lactose, 4 μg/ml erythromycin) after 48 h of incubation in anaerobiosis at 30 °C. Transformant ES3P was used for gene disruption (Biswas et al., 1993). Briefly, an overnight culture of strain ES3P grown in M17 broth containing erythromycin (4 μg/ml) at 30 °C was diluted 100-fold into fresh medium and incubated at 30 °C. When the culture reached an $O.D._{600\ nm}$ of 0.3–0.4, the growth temperature was shifted to 42 °C (a nonpermissive temperature for plasmid replication) until cells reached stationary phase. Dilutions of the culture were plated at 42 °C and erythromycin resistant colonies corresponding to pMI1 integrants appeared after 24 h of incubation. Integrants were screened using PCR and sequenced to verify the correct integration of pMI1 within the *ureE* gene. After this first integration step, the ES3PI derivative was grown in M17 broth at 30 °C until the stationary phase. The use of the permissive temperature of 30 °C stimulated the replication of the integrated plasmid and the second homologous recombination event that determined pMI1 excision and *ureE* gene replacement. Dilutions of the culture were plated at 42 °C and resolved integrants appeared as erythromycin sensitive colonies after 24 h of incubation. The erythromycin sensitive clones were PCR screened and sequenced to verify the effective replacement of $ureE_{DSM20617}$ with $ureE_{MIMO1}$. A further verification of the gene replacement was carried out by restriction analysis of the amplified *ureE* gene with *Sau*3A1 because the single base mutation in *ureE* of strain MIMO1, which determined a substitution of Asp_{29} with Asn_{29} in UreE, resulted also in the loss of one of the two *Sau*3A1 restriction sites present in *ureE*. Moreover, a further confirmation of *ureE* gene replacement in the genetic background of strain DSM 20617^T, was verified by *Rsa*I restriction of amplified *epsCD* genes as previously reported (Mora et al., 2002). Restriction digestion of the amplified *ureE* fragments was carried out for 5 h at 37 °C in 20 μl reaction mixture prepared according to *Sau*3A1 or *Rsa*I manufacture instruction (New England Biolabs, Ipswich, MA, United State). Restriction digests were subsequently analyzed by agarose electrophoresis (3 % *w/v* agarose gel, 0.2 μg/ml ethidium bromide, 40 mM Tris-acetate, 10 mM EDTA, pH 8,3). The gels were run at 5 V cm^{-1} in the appropriate buffer and photographed in UV light. Sequence of the urease gene locus of recombinant ES3 was carried out as previously reported (Della Scala et al., 2019; Mora et al., 2004).

Table 1
Bacterial strains and plasmids.

Strains, plasmid	Description	Reference
Escherichia coli VE7108	Km^R, containing the wildtype *repA* plasmid gene (not thermosensitive), host for pG$^+$host9 DNA manipulation	(Mora et al., 2004)
Streptococcus thermophilus		
MIMO1	Em^S, urease-positive $NiCl_2$-dependent, amino acid substitutions in UreE (Asp_{29} > Asn_{29}) and ureQ (Asp_{270} > Gly_{270})	(Mora et al., 2002; Della Scala et al., 2019)
DSM 20617^T	Em^S, urease-positive, wildtype	(Mora et al., 2004)
A16	Em^S, urease-negative, DSM 20617^T (*ΔureC3*)	(Mora et al., 2004)
ES3P	Em^R, urease-positive, carrying pMI1	This study
ES3PI	Em^R, urease-positive, *ureE*::pMI1	This study
ES3	Em^S, urease-positive, DSM 20617^T ($ureE_{MIMO1}$)	This study
pG$^+$host9	Em^R, thermosensitive replicon	(Maguin et al., 1996)
pMI1	pG$^+$host9 containing $ureE_{MIMO1}$	This study

2.3. In-gel detection of urease activity

Urease activity was visualized on native polyacrylamide gel as previously described (Mora et al., 2005). In specific, total bacterial proteins were extracted from cells collected by centrifugation from 200 ml of M17 culture grown at 37 °C for 12 h. Cells were washed twice in 50 mM sodium phosphate buffer (pH 7.5) and resuspended in 3 ml of the same buffer. Dithiothreitol was added to the cell suspension at a final concentration of 20 mM. Cell disruption was then carried out in a French Press (SLM Instrument, Rochester, NY, United States) and the resulting cellular extract was centrifuged at 20000 *g* for 30 min. The supernatant was concentrated approximately fivefold using a centrifugal filter device, Microcon YM50 (50,000 Da nominal molecular weight limit) (Millipore Corporation, Bedford, MA, United States) at 4 °C. During concentration, the protein solution was washed with 50 mM potassium phosphate buffer (pH 7.5). Total protein was evaluated using the Bradford method (Bradford, 1976). Total cell protein extract (10 μg) was analyzed with a 7 % polyacrylamide gel under non-denaturing conditions in a Mini-Protean III system apparatus (BioRad, Hercules, CA, United States). Electrophoresis was performed at 100 V in a Tris–glycine buffer system containing Tris– HCl (25 mM) and glycine (250 mM) at pH 8.8. Thioglycolic acid was added to the cathodic running buffer at a final concentration of 100 μM. After electrophoresis, the gel was washed three times in cold acetate buffer (5 μM) and once in distilled water. The

gel was then covered with 25 ml of a freshly prepared staining solution containing urea (0.10 M), p-nitroblue tetrazolium (Sigma–Aldrich, St Louis, MO, United States) (0.08 %), 1,4-Dithiothreitol (0.5 mM) and incubated at 37 °C, from 1 to 12 h, until visualization of dark blue bands. After staining, the reaction was stopped by a 5 min incubation in HCl (20 mM). A gel image was then captured using a digital camera (CoolPix 990, Nikon, Nital S.p.A., Torino, Italy).

2.4. Quantification of urease activity

Urease activity was measured in *S. thermophilus* cells using the phenol red assay described by Lanyi (1988) with some modifications. In detail, *S. thermophilus* cells were collected and quantified by flow cytometry using an Accuri C6 Plus apparatus (Becton Dickinson Biosciences, Milan, Italy) according to ISO 19344 IDF 232 (2015) as previously described (Martinović et al., 2023). Cell suspensions were standardized to a final cell density of 10^7 live cells/ml (measured as Active Fluorescence Units/ml, AFU/ml) in a urease mix solution prepared using 30 µl of solution A (2 g of urea dissolved in 2 ml of ethanol and 4 ml of sterilized deionized water) and 470 µl of solution B (1 g/l KH2PO4, 1 g/l K2HPO4, 5 g/l NaCl, 20 µg/ml phenol red). The urease-mix solution was aliquoted (150 µl) in 96 well microtiter plates and incubated at 37 °C for 6 h. Development of a red-violet color due to the release of ammonia by urease was monitored using a spectrophotometer EON (Biotek, Winoosky, VT, United States) that was programmed for readings ($O.D._{555nm}$) every 15 min for 6 h at 37 °C. At the end of the incubation, the urease activity expressed as maximum velocity ($mO.D._{555nm}$/min) was calculated using the software Gene5 (Biotek, Winoosky, VT, United States). Urease activity was also measured on total protein cell-free extract. To this aim, *S. thermophilus* cells collected by centrifugation and washed in Phosphate Buffered Saline (PBS) solution (0.15 M NaCl, 1 mM KH_2PO_4, 3 mM Na_2HPO_4, pH 7.4) and subjected to mechanical cell disruption carried out using a Precellys bead beater (Advanced Biotech Italia SRL, Seveso, Italy). The protein content of the total cell-free extract was determined using the Bradford method. For the evaluation of urease activity, the total cell-free extract containing 10 µg of protein was diluted in the urease mix solution, and the development of a red-violet color was monitored as above detailed. At the end of the incubation, the urease activity was expressed as maximum velocity per gram of protein ($mO.D._{555nm}$/min $g_{protein}$).

2.5. Transcription analysis

The total RNA was extracted from *S. thermophilus* cells that were collected at different time points during the growth in M17 (2 % *w/v* lactose), at 37 °C according to Arioli et al. (2010). A quantity of 1 µg of RNA was used for cDNA synthesis by iScriptTM cDNA Synthesis (Bio-Rad, Laboratoires, Milano, Italy). RT-qPCR was performed using 5 µl of cDNA solution in a total volume of 20 µl using the EvaGreenTM kit (BioRad, Laboratoires, Milano, Italy). The expression levels ureC, the primary subunit of urease was normalized using *pgk*, which codes for phosphoglycerate kinase (*pgk*), as the reference gene. For each condition, the measures were performed in triplicate with cDNA synthesized from two independent RNA samples. The real-time PCR was performed using the EvaGreenTM PCR master mix (BioRad, Laboratoires, Milano, Italy) as recommended. PCRs were performed in triplicate and run on a CFX96 instrument (BioRad, Laboratoires, Milano, Italy). Data were recorded as threshold cycles (CT), expressed as the mean ± standard deviation, analyzed using the BioRad CFX ManagerTM software and expressed as normalized expression (ΔΔCT) ± the standard error of the mean. The primer sets used in the real-time PCR experiments are the following: for *ureC* (ureCF GCTGACGGTAGTAATGCAACAA; ureCR GTGTGTCTTAATTCCTGCTGCA), for *pgk* (pgkF GTTACCGATTTCGATACCTTGCGC; pgkR TCCAGAACGTCCATTCGTGGCTAT).

2.6. Milk acidification rate and fermentation efficiency quantification

Acidification rates of wildtype and recombinants were evaluated in sterilized re-constituted skimmed milk supplemented with of 0.2 g/l of urea (filter-sterilized and added after milk sterilization) and or 5 µM $NiCl_2$. *S. thermophilus* cells were collected after growth in M17 broth (18 h, 37 °C), washed in PBS solution and the cell density was standardized by flow cytometry, as described above, to inoculate 20 ml of pre-heated skimmed milk at a final cell density of 10^7 AFU/ml. Incubation of milk cultures was carried out at 37 °C until reaching pH 5. The pH of milk cultures was measured continuously and recorded every 1 min using an iCINAC system (AMS, Guidonia, Italy). Data were reported as the average of three replicates with the relative standard deviation. The first time derivative (dpH/dt) was also reported to better identify the evolution of urease activity as increase of dpH/dt as reported by Pernoud et al. (2004).

Measurement of fermentation efficiency was based on evaluation of the homolactic fermentation yield (*yHlf*), the glycolytic yield (*yGly*) and the lactose intake capacity (*cLacIn*), which have been calculated as previously reported (Arioli et al., 2022). In specific, *yHlf* was calculated as the % of L-lactic acid produced on the L-lactic acid expected to be synthesized based on the amount of lactose consumed considering that only the glucose moiety of lactose is metabolized in *S. thermophilus*, *yHlf* = L-lactic acid (mM) / [lactose consumed (mM) x 2] x 100. *yGly* was calculated as the % of glucose catabolized on the glucose expected to be catabolized based on the amount of lactose consumed, *yGly* = [100 – (glucose (mM)/lactose consumed (mM) x 100]. *cLacIn* was calculated as the % of lactose consumed on the total lactose measured in milk before *S. thermophilus* inoculum (lactose t0), *cLacIn* = (lactose consumed (mM) = lactose t0 (mM)) x 100. The lactose consumed was calculated as the difference between the lactose t0 and the residual lactose at the end of the incubation, lactose tf, lactose consumed (mM) = (lactose t0) - lactose tf). Lactose, glucose, and L-lactic acid quantification have been quantified enzymatically using the UV method for the determination of foodstuffs and other materials (R-Biopharm Italia Srl, Melegnano, Italy).

2.7. Statistical analysis

Data presented are the mean of at least three independent experiments. Results on gene expression and metabolites quantification for catabolic performance were analyzed by one-way ANOVA with a *post hoc* Tukey HSD test using GraphPad Prism software.

3. Results and discussion

3.1. ureE gene replacement in DSM 20617^T

A single base mutation in *ureE* determined a substitution of Asp_{29} with Asn_{29} in the metallochaperone in a high conserved region even if not in the region known to be involved in the binding of Ni ions (Fig. S1). A BLAST analysis revealed that substitution of Asp_{29} with Asn_{29} in *S. thermophilus* UreE is not unique for the strain MIMO1 because it is present in 35 records referring to as many *S. thermophilus* strains most of which isolated from yogurt samples (Table S1). These include, strains CNCM I-1630 (McNulty et al., 2011), CAG:236 (derived from human gut metagenome, CDA38230), and the IMAU strains (Zhao et al., 2021).

To investigate the role of the substitution of Asp_{29} with Asn_{29} in UreE, the *ureE* gene of the reference strain DSM 20617^T ($ureE_{DSM20617}$) was replaced by *ureE* gene of strain MIMO1 ($ureE_{MIMO1}$) by a double crossover event mediated by the pGhost$^+$ derivative pMI1 (Fig. 1) and screening of erythromycin-sensitive recombinants. The successful replacement of $ureE_{DSM20617}$ with $ureE_{MIMO1}$ in the genetic background of strain DSM 20617^T was verified by *Sau*3AI restriction analysis of *ureE*, and by *Rsa*I restriction analysis of *epsCD* genes (Fig. S2) and by sequencing of urease gene cluster from the recombinant ES3 (accession n. PP083786).

3.2. In-gel detection of urease activity in DSM 20617^T and in the recombinant ES3

The detection of urease activity of *S. thermophilus*, carried out in native PAGE, allowed the visualization of a complex enzymatic profile of activity as previously reported (Mora et al., 2005). The two *S. thermophilus* strains showed differences in number of signals and in their electrophoretic mobility, despite few sequence differences have been observed in urease genes (Della Scala et al., 2019). The zymograms highlighted several activity signals, whose number was strain-dependent and strongly influenced by $NiCl_2$ supplementation in the culture medium. *S. thermophilus* DSM 20617^T showed three activity signals designated **a**, **b** and **c** (Fig. 2A) only when cells were grown in $NiCl_2$ supplemented M17. In absence of $NiCl_2$ supplementation, the urease activity was only slightly detected in-gel (Fig. 2C). Conversely, *S. thermophilus* MIMO1 showed four clear activity signals designated **a**′, **b**, **c** and **d** (Fig. 2A) only when cells were grown in $NiCl_2$ supplemented M17. In absence of $NiCl_2$ supplementation, as expected, urease activity was not detectable in-gel (Fig. 2B). The observed small differences in the zymogram profile of MIMO1 with $NiCl_2$ could be due to minimal differences in the physiological state of harvested *S. thermophilus* cells and/or to other proteins/molecules present in the genetic background of strain MIMO1 that could interfere with the electrophoretic mobility of urease proteins and therefore in the in-gel detection of the urease activity. Such differences in the zymogram profile were never detected in the genetic background of strain DSM 20617^T.

As previously reported (Mora et al., 2005), the presence of multiple activity signals in the zymogram analysis of *S. thermophilus* could be explained by the co-existence of several quaternary structures of the three main urease subunits α, β and γ, or by the presence of complexes between active urease and some accessory proteins. Interestingly the replacement of *ureE*$_{DSM20617}$ with *ureE*$_{MIMO1}$ in strain DSM 20617^T consistently affected the urease activity zymogram of the recombinant strain ES3 (Fig. 2C) which appeared composed of the clearly detectable

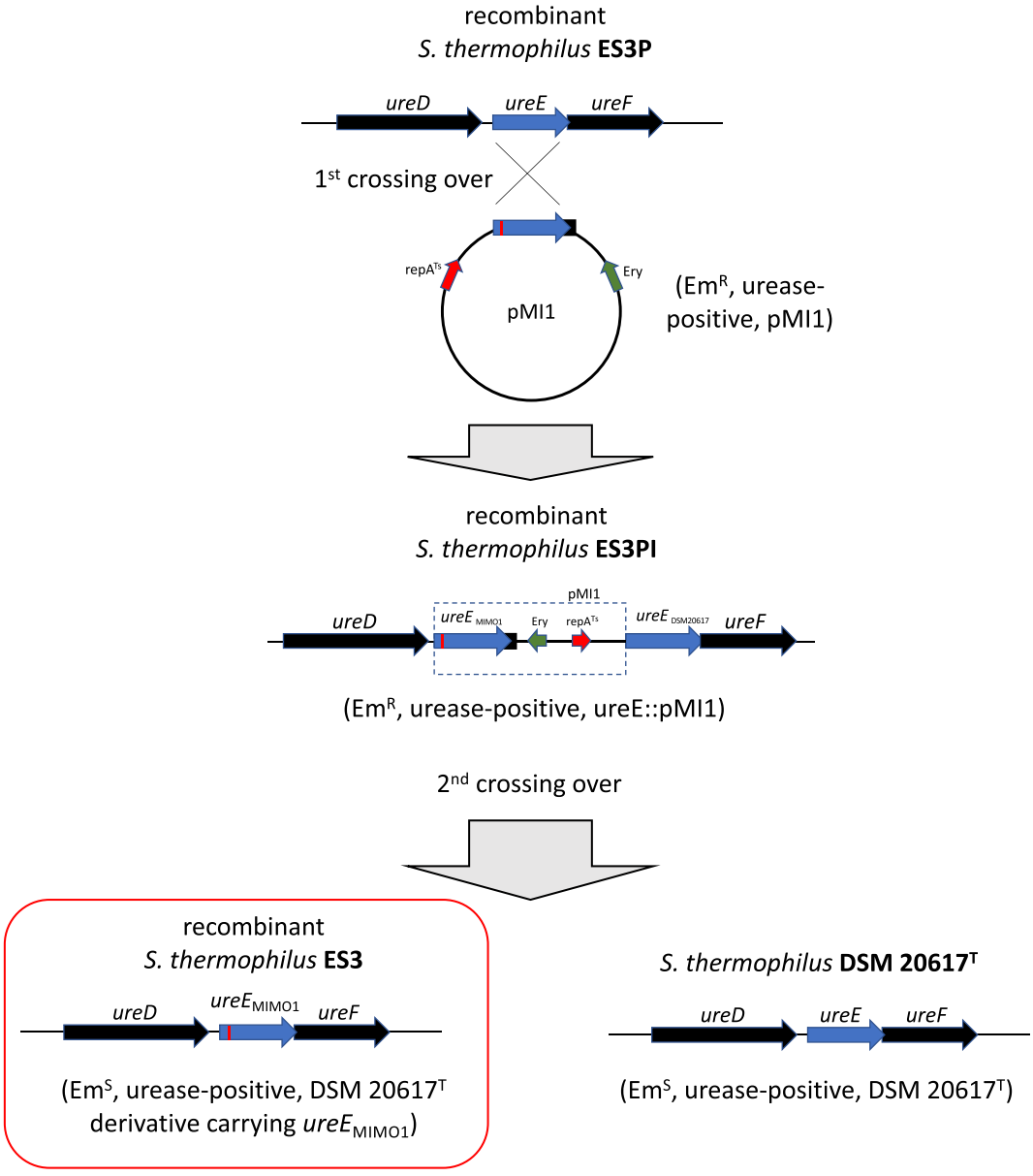

Fig. 1. Schematic description of the gene replacement strategy applied to obtain the recombinant ES3 by double event of homologous recombination (1st and 2nd crossing over) mediated by pMI1 a derivative of pG$^+$host. The chromosomal region of *S. thermophilus* DSM 20617^T encompassing *ureD*, *ureE* and *ureF* genes is represented. pMI1 contains *ureE*$_{(MIMO1)}$ as a blue arrow with a vertical red line indicating the single base mutation that determines a substitution of Asp_{29} with Asn_{29}. The sensitivity of recombinant strains to erythromycin and the urease phenotype are indicated in figure.

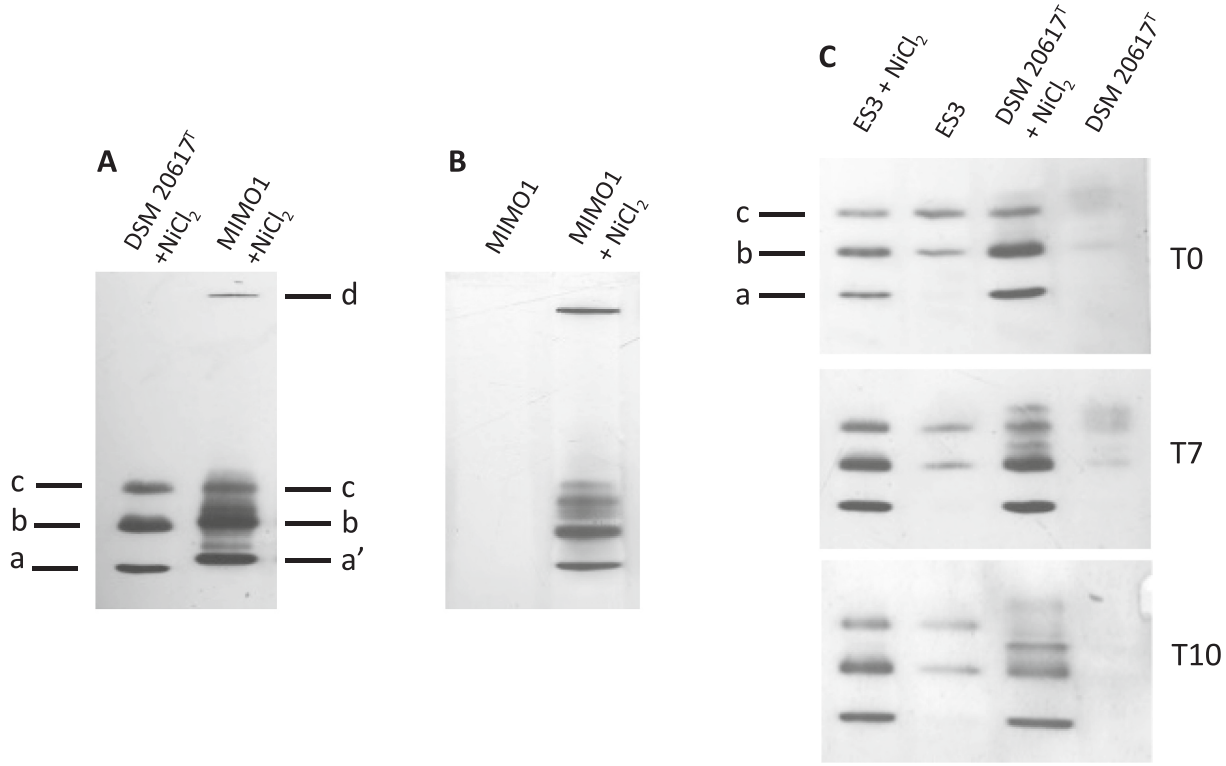

Fig. 2. In-gel detection of urease activity in the total cell protein extract of *S. thermophilus* DSM 20617^T, MIMO1 and ES3. (A) Urease activity electrophoretic profiles obtained from cells grown for 18–24 h at 37 °C in M17 broth supplemented with 5 μM $NiCl_2$. (B) Urease activity electrophoretic profiles obtained as previously described for strain MIMO1 grown without and with $NiCl_2$ medium supplementation. (C) Urease activity electrophoretic profiles obtained as previously described for the recombinant ES3 and the wildtype DSM 20617^T without and with $NiCl_2$ medium supplementation immediately after total cell protein extraction, T0 and after storing the total cell protein extract at 4 °C for 7 days, T7, and 10 days T10. Based on their electrophoretic mobility the urease activity signals are designated **a**, **a′**, **b**, **c** and **d** and indicated in figure.

activity signals **b** and **c**, even in absence of $NiCl_2$ supplementation in the growth medium (Fig. 2C). The additional signal **a** appeared only when $NiCl_2$ was available (Fig. 2C). By comparing the urease zymogram of ES3 and wildtype DSM 20617^T, obtained without $NiCl_2$ supplementation, it can be hypothesized that the replacement of Asp_{29} with Asn_{29} in UreE resulted in a significant increase in urease activity in recombinant ES3 compared to the wild type (Fig. 2C). Furthermore, a more stable signal of gel-detected urease activity was observed for ES3 during storage at 4 °C of total protein cell extracts, compared to wildtype, thus suggesting that the replacement of Asp_{29} with Asn_{29} in UreE also resulted in greater stability of the ES3 urease complexes.

3.3. Quantification of urease activity and transcriptional analysis of *ureC* in DSM 20617^T and in the recombinant ES3

Urease activity was quantified on total cell extracts obtained from cell collected during the exponential phase of growth of the wildtype and the recombinant ES3. The results obtained (Table 2) showed a significantly higher level of urease activity in the recombinant ES3 compared to the wildtype, confirming results of the zymogram analysis. However, the higher level of urease activity quantified in the recombinant ES3 was not supported by the increase of transcription of the urease operon. Indeed, there were no statistically significant differences in expression level of *ureC* gene both in the wildtype and in the recombinant ES3 (Table 2). It could be therefore hypothesized that the differences in urease activity highlighted between the wildtype and the recombinant ES3 are directly consequence of the replacement of Asp_{29} with Asn_{29} in the UreE accessory protein.

Table 2
Urease activity and transcriptional analysis of *ureC* gene.

Sampling time	Urease activity (mO.D.$_{555nm}$/min $g_{protein}$)		*ureC* normalized fold expression (ΔΔCT)	
	DSM 20617^T	ES3	DSM 20617^T	ES3
1.5 h	0.77 ± 0.09^a	1.1 ± 0.1^b	1.2 ± 0.4^a	0.74 ± 0.08^a
2.5 h	0.56 ± 0.07^a	1.3 ± 0.1^b	0.7 ± 0.1^a	0.60 ± 0.07^a
3.0 h	1.79 ± 0.06^a	3.3 ± 0.4^b	1.1 ± 0.3^a	1.4 ± 0.6^a
3.5 h	1.96 ± 0.06^a	3.6 ± 0.2^b	3.4 ± 1.3^a	3.0 ± 0.4^a

When mean ± SD values in a row are accompanied by the same letter, it indicates that there are no statistically significant differences ($p > 0.05$), otherwise it indicates that there are statistically significant differences ($p < 0.05$).

3.4. Milk acidification rate of *S. thermophilus* strains and related recombinants

With the aim to verify the effect of urease activity of *S. thermophilus* strains and related recombinants on milk acidification, pH was monitored continuously during the growth using an iCINAC system. In this context, the recombinant strain A16, a DSM 20617^T (*ΔureC3*) derivative with a 693 bp in-frame deletion in *ureC* gene (Mora et al., 2004) (Table 1), was used a urease-negative control in comparison with the wildtype and the new recombinant ES3. Milk acidification was also monitored for strain MIMO1 without and with $NiCl_2$ supplementation. In all condition tested the reconstituted skimmed milk was supplemented with 0.2 g/l urea, an amount of urea that is like what is naturally present in milk (Butler et al., 1995). The results obtained clearly showed that in presence of urease activity, milk acidification is strongly impacted determining a delay in reaching the reference value of pH 5.2 (Fig. 3A, B). This delay is clear by comparing the acidification curves of DSM 20617^T and the derivative urease negative mutant A16. Analogously, the activation of urease activity in MIMO1 by $NiCl_2$

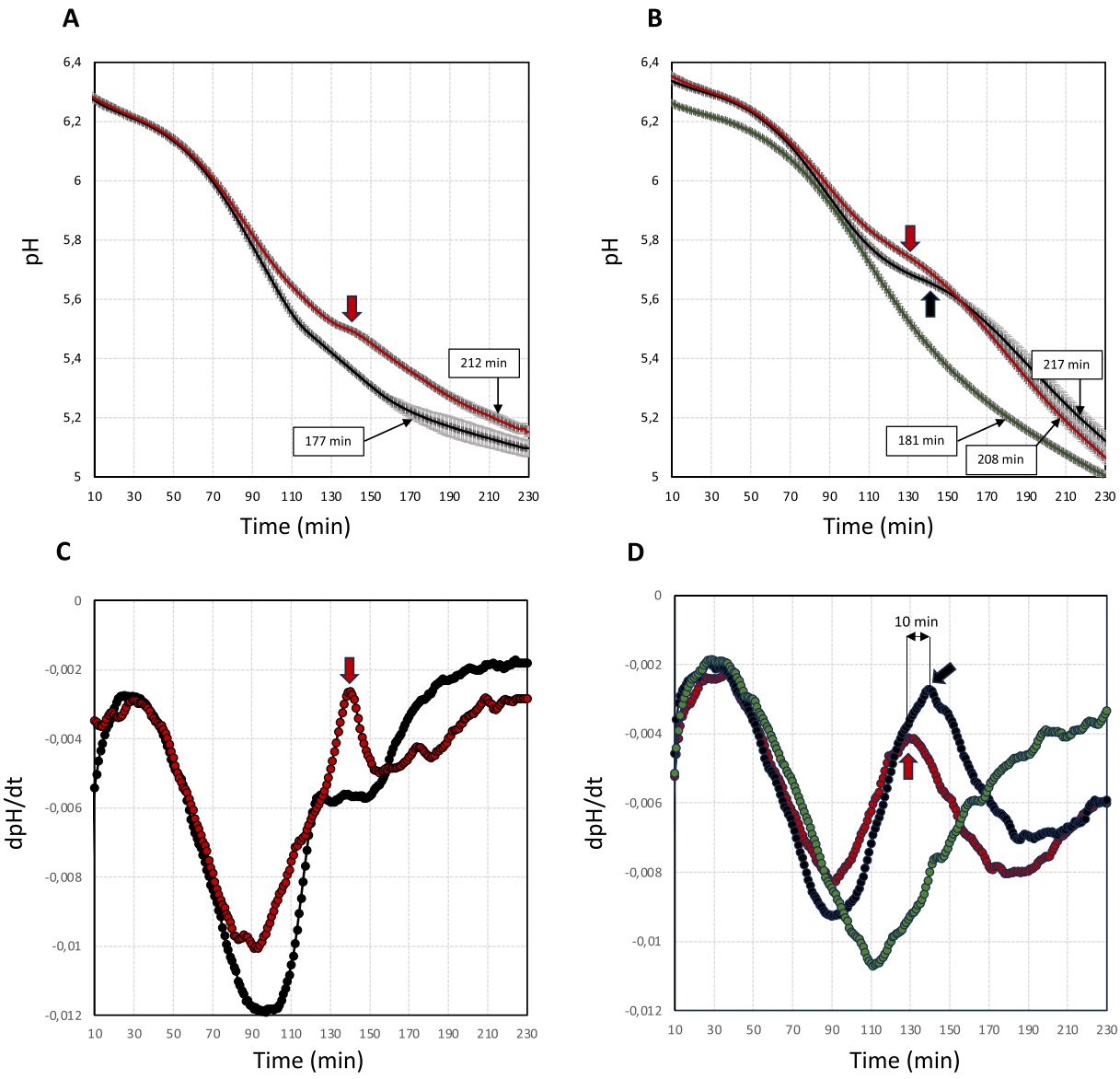

Fig. 3. Acidification curves (pH *vs* time) (A, B) and the relative first time derivative (dpH/dt *vs* time) (C, D) in reconstituted pasteurized skimmed milk supplemented with 0.2 g/l urea of *S. thermophilus* DSM 20617^T, MIMO1, ES3 and A16. (A, C) Acidification curves and first time derivative of strain MIMO1 in absence (black line) or in presence (red line) of 5 μM $NiCl_2$ supplementation. (B, D) Acidification curves and first time derivative of strain DSM 20617^T (black line), the derivative urease-negative A16 (green line) and the recombinant ES3 (red line). The time to reach the reference pH value of 5.2 is showed in the plots A and B. The arrows indicate the decrease of acidification due to urease activity. Acidification curves data are reported as the means and SD of three independent analysis.

supplementation resulted in a 35 min delay in reaching pH 5.2. Analogously, the urease-positive DSM 20617^T reached pH 5.2, 36 min later than its urease-negative mutant A16. By contrast, the milk acidification rate of DSM 20617^T and its derivative ES3 were very close even if the reference pH 5.2 was reached 8 min before by ES3 compared to the wildtype. Interestingly, the well-known decrease of acidification determined by the ammonia released by urea hydrolysis it is anticipated by ES3 compared to the wildtype. This phenomenon is more evident by analyzing the first time derivative (dpH/dt) (Fig. 3C, D) where the maximum level of decrease of acidification is identified with a sharp peak. This peak is evident for strain MIMO1 in milk supplemented with $NiCl_2$, whereas it is absent for strain MIMO1 in milk without $NiCl_2$ supplementation and for the urease negative mutant A16. Interestingly, by comparing the strain DSM 20617^T and its derivative ES3, the two peaks are not over imposed but 10 min shifted thus confirming that urease activity of ES3 is anticipated compared to the wildtype. Moreover, the maximum value of dpH/dt reached by ES3 is lower compared to that reached by the wildtype, thereby indicating, as expected, that the highest level of urease activity was shown by the recombinant ES3.

3.5. *Catabolic performance in milk of Streptococcus thermophilus strains and related recombinants*

As reported in previous studies, urease activity impact consistently on the energetic metabolism of *S. thermophilus* and specifically on the homolactic fermentation (Arioli et al., 2010; Arioli et al., 2017; Yamauchi et al., 2019; Pernoud et al., 2004; Yu et al., 2020). More recently, using as model system energetically discharged cells resuspended in lactose solution, we reported that urease activity, such as NH_3, positively affected the *yHlf* and the *yGly* while negatively affected the *cLacIn* (Arioli et al., 2022). Here, using as samples the milk cultures previously obtained to evaluate the milk acidification rate, lactose, glucose, lactic acid has been quantified enzymatically to allow the calculation of *yHlf*, *yGly* and *cLacIn*. The results obtained (Table 3)

Table 3
Lactose consumed, glucose, galactose, lactic acid quantification and calculation of the catabolic performance in reconstituted skimmed milk supplemented with 2 mM urea.

Strain and culture condition	Metabolites (mM)				Catabolic performance		
	Lactose	Glucose	Galactose	L-lactic acid	Homolactic fermentation yield (*yHlf*) (%)	Lactose intake capacity (*cLacIn*) (%)	Glycolytic yield (*yGly*) (%)
DSM 20617^T	21 ± 2^a	3.0 ± 0.3^c	22.0 ± 0.6^e	8.1 ± 0.6^i	19.2	13.7	86.0
A16	23 ± 2^a	4.6 ± 0.3^d	19.6 ± 0.6^f	5.2 ± 0.6^l	11.0	15.2	80.3
ES3	22 ± 5^a	3.5 ± 0.3^c	22.0 ± 0.8^e	9.3 ± 1.3^i	20.6	14.6	85.0
MIMO1	30 ± 1^b	3.0 ± 0.3^c	29.0 ± 0.3^g	19.2 ± 0.6^m	31.8	19.7	90.2
MIMO1, $NiCl_2$*	32 ± 2^b	3.1 ± 0.3^c	31.8 ± 0.4^h	25.0 ± 3.0^n	39.2	20.5	90.1

* *S. thermophilus* MIMO1 grown in M17 (2 % lactose) supplemented with 5 μM $NiCl_2$ used to inoculate reconstituted skimmed milk supplemented with 5 μM $NiCl_2$.
When mean ± SD values in a column are accompanied by the same letter, it indicates that there are no statistically significant differences ($p > 0.05$), otherwise it indicates that there are statistically significant differences ($p < 0.05$).

showed that the urease-negative A16 produced significantly less lactic acid than the wildtype DSM 20617^T while excreting a higher amount of glucose, strongly supporting that delating urease activity in *S. thermophilus* has the effect of reducing the *yHlf* (Arioli et al., 2022). Therefore, the wildtype showed higher values of *yHlf* and *yGly* a lower *cLacIn* than its urease-negative derivative. Similarly, in strain MIMO1 the activation of urease by $NiCl_2$ supplementation resulted in *yHlf* and a decrease of *cLacIn*, but in comparable values of *yGly*. Conversely, the data related to lactose, glucose and lactic acid quantification obtained from milk cultures of DSM 20617^T and its derivative ES3 were not significantly different, thus suggesting that the increase of urease activity obtained in the recombinant ES3 by replacing Asp_{29} with Asn_{29} in the metallochaperone UreE is not sufficient to affect the catabolic performance of the strain in milk.

4. Conclusion

Urease activity of *S. thermophilus* impacts significantly the physiology of this species being involved in several biosynthetic pathways (Arioli et al., 2007; Arioli et al., 2009a; Arioli et al., 2009b; Monnet et al., 2005) and affecting homolactic fermentation (Arioli et al., 2010; Arioli et al., 2022; Della Scala et al., 2019; Yamauchi et al., 2019; Monnet et al., 2004; Mora et al., 2004; Pernoud et al., 2004; Yu et al., 2019). Urease-negative strains are rare within the species *S. thermophilus*, however some exception occurs, such as the strain MIMO1 which is considered a urease-positive Ni-dependent strain. The strain MIMO1 is urease-negative most probably because of a defective Ni intake due to a mutation in *ureQ* gene coding for a membrane permease (Della Scala et al., 2019). However, when the strain MIMO1 is cultivated in medium supplemented with $NiCl_2$ it showed a high level of urease activity. Interestingly, the strain MIMO1 also showed a mutation in *ureE* gene which encodes for a metallochaperone involved in Ni delivery to the urease catalytic site. To investigate the possible effect on urease activity of the substitution of Asp_{29} with Asn_{29} in the metallochaperone UreE, *ureE* gene of the reference strain DSM 20617^T was replaced by *ureE* gene of strain MIMO1 to obtain the recombinant ES3. The obtained recombinant ES3 was therefore considered isogenic to DSM 20617^T apart from the UreE substitution of Asp_{29} with Asn_{29}.

The results obtained by the phenotypic and genotypic characterization of the recombinant ES3 in comparison with the wildtype, clearly highlight that the single amino acid substitution in UreE determined a higher stability of urease complexes and an increase of urease activity irrespectively of the transcription of urease genes. Therefore, it was hypothesized that the increase of urease activity was due a more efficient assembly of an active urease complex which represents a novelty in the field.

Because of the known role of urease activity on *S. thermophilus* milk fermentation performance, the DSM 20617^T wildtype and the derivative mutants, and the strain MIMO1, have been tested in milk acidification assays. The results obtained confirmed that urease activity determined a delay in milk acidification due to the buffering effect of ammonia, generated by urea hydrolysis, on the lactic acid produced by the homolactic fermentation. The substitution of Asp_{29} with Asn_{29} in the metallochaperone UreE determined only an anticipation of urea hydrolysis in milk of the recombinant ES3 compared to the wildtype, without affecting its milk acidification performance in terms of *yHlf*, *cLacIn* and *yGly*.

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CRediT authorship contribution statement

Stefania Arioli: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicola Mangieri:** Formal analysis, Data curation. **Ylenia Zanchetta:** Formal analysis, Data curation. **Pasquale Russo:** Writing – review & editing, Data curation. **Diego Mora:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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