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ORIGINAL ARTICLE

# Subtilisin 6 From the Dermatophyte *Trichophyton benhamiae* Is a Marker of Infection but Not a Unique Virulence Factor

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## ABSTRACT

**Background:** *Trichophyton benhamiae* is a common dermatophyte whose natural host is the guinea pig and which causes highly inflammatory skin lesions in humans. The subtilisin 6 (SUB6) of this fungus belongs to a family of 12 *SUB* genes. Its encoding gene, overexpressed in vivo but not in vitro, has been considered a potentially important virulence factor, but its role in pathogenesis remains to be elucidated.

**Objectives:** The aim of this study was to assess the role of *T. benhamiae* SUB6 in virulence in a mouse skin infection model.

**Methods:** To assess the contribution of SUB6 to virulence, *SUB6*-deleted (ΔSUB6) and complemented strains were generated by genetic transformation. The pathogenicity of these strains was compared with that of the parental strain in vivo in mice, based on the evolution of skin symptoms, histopathological lesions and molecular analyses targeting the expression of host pro-inflammatory genes and fungal genes encoding subtilisins from the same family as SUB6.

**Results:** The ΔSUB6 strain induced superficial skin signs and histopathological inflammatory lesions similar to those caused by the parental strain. Significant overexpression of the *SUB1*, *SUB3*, *SUB8* and *SUB10* genes in the tissues was observed regardless of the strain tested, with no difference between these strains, reflecting the absence of any compensatory mechanism among subtilisins.

**Conclusions:** SUB6 appears to be more of a marker of fungal infection than a virulence factor, at least acting alone.

**Abbreviations:** ANOVA2, 2-way analysis of variance; ATCC, American Type Culture Collection; BD2, β-defensin 2; CFU, colony-forming unit; FRT, flippase recombinase-recombination target; IHEM, Institute of Hygiene and Epidemiology Mycology; IL, interleukin; *IL*, interleukin gene; MEP, metalloprotease protein; *MEP*, metalloprotease gene; mRNA, messenger RNA; PAS, periodic acid-Schiff; PI, post-infection; qPCR, quantitative PCR; RHE, reconstructed human epidermis; RT, reverse transcription; SDA, Sabouraud dextrose agar; SUB, subtilisin protein; *SUB*, subtilisin gene; UTR, untranslated region; WT, wild-type.

## 1 | Introduction

Dermatophytoses are the most widespread mycoses in humans and animals worldwide, with an estimated prevalence of 20%–25% [1–3]. The genomes of the causative agents, dermatophytes, harbour a large repertoire of genes encoding hydrolytic enzymes, in particular proteases, which show a high degree of similarity from one species to another. A similar repertoire is also observed in *Aspergillus* species, with the notable difference that the genes encoding secreted endoproteases have developed in dermatophytes, forming true families of proteases such as the one comprising 12 genes encoding secreted subtilisins (SUB) [4]. As dermatophytes are almost exclusively localised in keratinized tissues, secreted proteases, including keratinolytic ones, have long been considered as virulence factors [5, 6], for example SUB3, which is involved in the adhesion of the zoophilic *Microsporum canis* to the epidermis [7–10].

Among other common zoophilic dermatophytes, *Trichophyton benhamiae*, whose natural reservoir is the guinea pig, is an emerging zoonotic species which causes highly inflammatory lesions in humans [11, 12]. In order to characterise the *T. benhamiae* secretome during infection, a complete gene expression profile of the fungus was obtained during infection of its natural host by RNA-seq analysis [13]. Strikingly, the genes encoding most major proteases secreted by the fungus in vitro were found not to be expressed in vivo, and conversely, the genes encoding most of the proteases secreted by the fungus during infection were found not to be expressed in vitro. The most highly upregulated gene encoding a secreted protease during infection was that encoding a subtilisin, namely SUB6. Similarly, the *SUB6* gene was also found to be overexpressed in mice infected with *T. benhamiae* [14]. This subtilisin is the ortholog of the major *Trichophyton rubrum* allergen Tri r2, which causes skin reactions in patients with asthmatic sensitisation to *Trichophyton* [15]. Subsequent proteomic analysis of *T. rubrum*-infected nails also revealed SUB6 as the major protein secreted by this fungus in cases of onychomycosis [16, 17]. SUB6 was also detected in reconstructed human epidermis (RHE) infected with *T. rubrum* [14], as well as in guinea pig skin explants infected ex vivo with *T. benhamiae* [18] and in bovine ringworm with *T. verrucosum* [19]. These results support the potential role of the SUB6 protein as a virulence factor and/or a marker of infection.

Herein, we evaluated the pathogenic role of the *T. benhamiae* SUB6 endoprotease in a recently optimised mouse model of infection. As the strain deleted at the subtilisin 6 locus infected and invaded tissues in the same way as the parental strain during dermatophytosis, this study demonstrated that subtilisin 6 is not essential for the initiation or development of *T. benhamiae* infection. The strong overexpression of the *SUB6* gene observed here and in other infection models suggests a significant role during the infectious process, but simple mutagenesis may not be sufficient to study the precise role of this protease. Subtilisin 6 is therefore a strong fungal marker of infection but is not a virulence factor per se.

## 2 | Materials and Methods

### 2.1 | Dermatophyte Strains and Genetic Manipulation

The *T. benhamiae* strain IHEM 20161 (LAU 2354-2; CBS 112371; ATCC MYA-4681) was originally isolated from a human with highly inflammatory tinea faciei in contact with a guinea pig [12]. Its genome has been sequenced and annotated [20]. In addition, IHEM 20161 was proved to be suitable for targeted gene deletion [21, 22], and was used for *RNA-Seq* analysis of the fungus during infection as well as under different in vitro growth conditions [13]. This strain was therefore used to produce subsequent *T. benhamiae* mutants listed in Table 1, all maintained on Sabouraud dextrose agar (SDA; BD Bioscience). The *T. benhamiae* KU70-lacking mutant (ΔKU70), which was produced from the wild-type (WT) strain IHEM 20161 (Figure S1), was used as the recipient strain for genetic manipulation. Microconidium formation was induced at 28°C using 1/10 SDA supplemented with 500 μg/mL cycloheximide (Wako Chemical) and 50 μg/mL chloramphenicol (Wako Chemical). *Agrobacterium tumefaciens* EHA105 was maintained and used as a mediator in the transformation system as previously described [23]. *Escherichia coli* DH5α (Nippon Gene) was used for molecular cloning.

#### 2.1.1 | Total DNA Extraction

Total DNA was extracted according to the method of Girardin and Latgé [24]. The growing mycelia from each dermatophyte strain were collected after incubation on SDA for three to 4 days at 28°C, frozen in liquid nitrogen, and ground twice with a multi-beads shocker (Yasui Kikai) at 2000 rpm for 10 s.

**TABLE 1** | Strains of *T. benhamiae* used and obtained in this study.

| Name of the strain | Abbreviation | Identification number | Genotype |
|---|---|---|---|
| Wild-type strain | WT | IHEM 20161 | / |
| Parental strain | ΔKU70 | ΔTbeKu70-5-1 (TIMM 40015; IFM 66349) | *KU70*-lacking mutant |
| *SUB6*-deleted strain | ΔSUB6 | ΔAbSUB6-14-5 | *KU70*-lacking and *SUB6*-lacking mutant |
| *SUB6*-complemented strain | SUB6c | AbSUB6C-23-3 | *KU70*-lacking and *SUB6*-lacking mutant, reintroduction of the *SUB6* gene |

*Note: Arthroderma benhamiae* (Ab) has been renamed *Trichophyton benhamiae* (Tbe); IFM: Medical Mycology Research Center of Japan; IHEM: Institute of Hygiene and Epidemiology Mycology; SUB: subtilisin; TIMM: Teikyo Institute of Medical Mycology.

### 2.1.2 | Construction of Vectors for Targeted-Gene Disruption

The SUB6-targeting vector was constructed from a binary vector pAg1-*AbKu70*/T2 (Figure S1; Table S2). Roughly 2.0 kb of the 5′-and 3′-untranslated regions (UTRs) of the *SUB6* gene (ARB_05307) were amplified from total DNA of the ΔKU70 strain by PCR with the P1–P4 and P5–P6 primer pairs, respectively (Table S1). Each fragment was double digested with the restriction enzymes SpeI/ApaI and BamHI/KpnI, respectively, and inserted into the corresponding sites within pAg1-*AbKu70*/T2 to obtain pAg1-*AbSUB6*/T (Figure 1; Table S2). For complementation of the *SUB6* gene in the *T. benhamiae* ΔSUB6 strain, an approximately 3.0 kb fragment containing the 5′-UTR and the coding region of the *SUB6* gene was amplified from total DNA of the ΔKU70 strain by PCR with the P7-P10 primers (Table S1). The resulting fragment was digested with SpeI/ApaI and inserted at the incision site of pAg1-*AbSUB6*/T to generate pAg1-*AbSUB6*/C (Figure 1; Table S2).

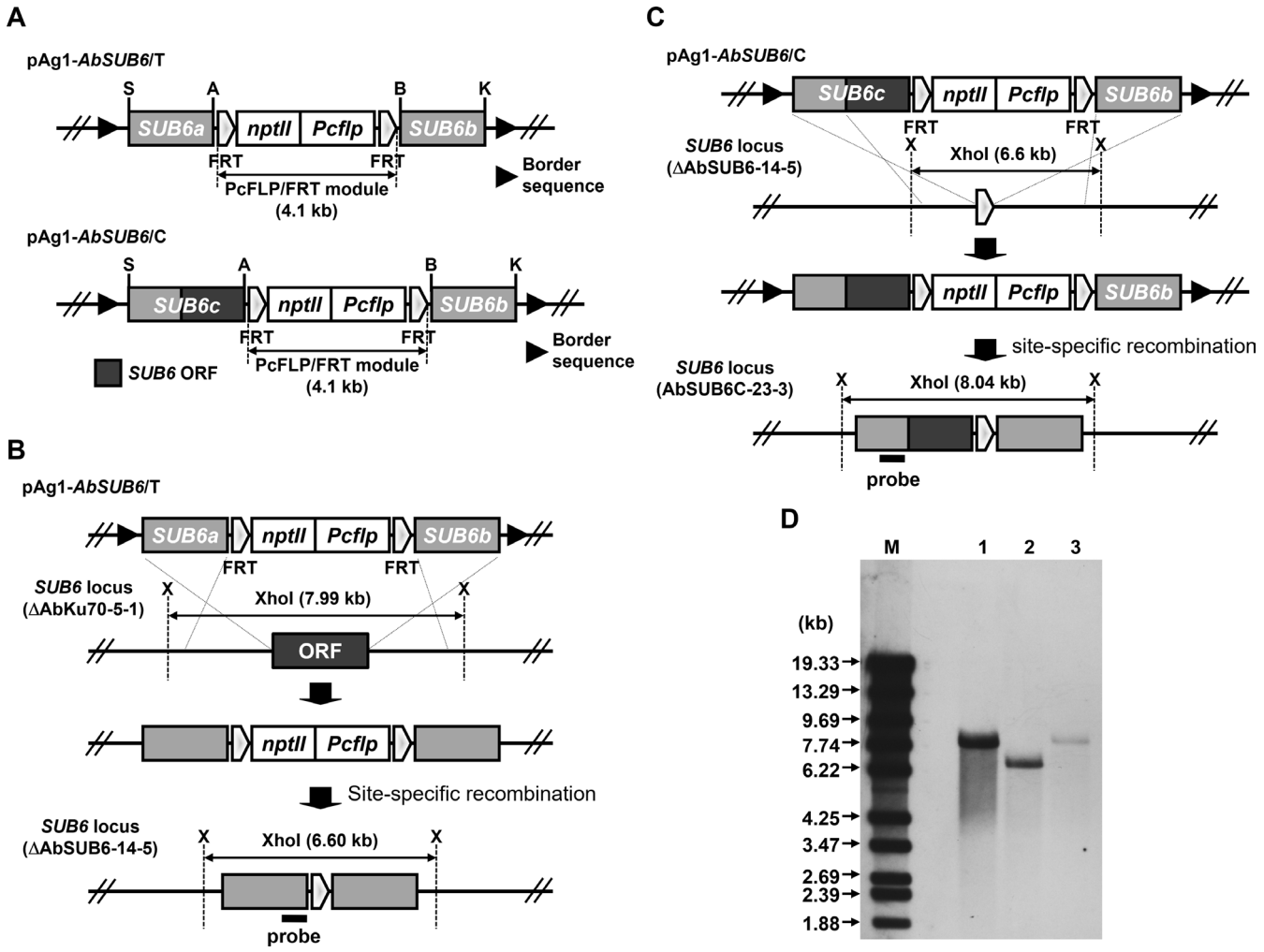


**FIGURE 1** | Disruption of the *SUB6* gene of *T. benhamiae* ΔTbeKu70-5-1 and subsequent reintroduction of the *SUB6* gene by a gene replacement strategy. (A) Schematic representation of the binary SUB6-targeting vector pAg1-*AbSUB6*/T and the binary *SUB6*-complementation vector pAg1-*AbSUB6*/C. Border sequences are the specific regions that delineate the DNA to be transferred during *A. tumefaciens*-mediated transformation. FRT, flippase (FLP) recombinase-recombination target. Restriction enzyme sites: A, ApaI; B, BamHI; K, KpnI; S, SpeI. (B) Schematic representation of the *SUB6* locus before and after excision of the PcFLP/FRT module. Disruption of the *SUB6* gene was achieved by transformation of the recipient strain ΔAbKu70-5-1 with the PcFLP/FRT module from the pAg1-*AbSUB6*/T vector. The PcFLP/FRT module was then excised by site-specific recombination between the flanking FRT sequences via conditional expression of the *Pcflp* gene after transformation. Restriction enzyme site: X, XhoI. (C) Reintroduction of the *SUB6* gene by transformation of the recipient strain ΔAbSUB6-14-5 with the PcFLP/FRT module from the pAg1-*AbSUB6*/C vector. The PcFLP/FRT module was then excised by site-specific recombination between the flanking FRT sequences via conditional expression of the *Pcflp* gene after transformation. Restriction enzyme site: X, XhoI. (D) Verification of the deletion and subsequent reintroduction in the *SUB6* locus by southern blotting analysis. Aliquots of approximately 10 μg of genomic DNA from each strain were digested with *PstI* and separated by electrophoresis in a 0.8% (w/v) agarose gel. Lane 1, ΔAbKu70-5-1 (parental strain); lane 2, ΔAbSUB6-14-5; lane 3, AbSUB6c-23-3. A fragment of about 540 bp of the *SUB6* locus was amplified by PCR with the primers P17 and P18 (Table S1) and used as a hybridization probe. DNA standard fragment sizes are shown on the left.

#### 2.1.3 | Fungal Genetic Transformation

Genetic transformation of each *T. benhamiae* strain was performed by the *Agrobacterium tumefaciens*-mediated transformation method as described previously [25]. After co-cultivation, nylon membranes (Hybond-$N^+$) (Cytiva) were transferred onto SDA containing 250 μg/mL G418 (Wako) and 10 μM $CuSO_4 \cdot 7H_2O$ and overlaid with 10 mL of the same solution. For sufficient selection of transformants, the plates were further overlaid after 48 h with 10 mL of SDA containing 350 μg/mL G418 and 10 μM $CuSO_4 \cdot 7H_2O$, then incubated for 4–5 days. The colonies regenerating on the selective media were considered as putative G418-resistant clones and transferred onto MOPS-buffered RPMI 1640 agar medium supplemented with 500 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 200 μg/mL cefotaxime sodium (Sanofi-Aventis) (if necessary) and 20 μM bathocuproine disulfonate (BCS) (Dojindo Laboratories) and passaged several times. The *T. rubrum* ctr4 promoter (Pctr4) is a conditional promoter that is repressed in the presence of copper. Chelation of copper by bathocuproine sulfate activates the Pctr4, leading to the induction of the *Pcflp* gene expression. Expression of PcFLP recombinase leads to the excision of the selectable marker via PcFLP-mediated site-specific recombination between the flanking flippase recombinase-recombination target (FRT) sequences.

#### 2.1.4 | Screening of the Desired Transformants

The desired transformants were finally screened by PCR, Southern blotting analysis, and nucleotide sequencing. Aliquots of 50–100 ng of the total DNA were used as templates in the PCR reactions. For Southern blotting analysis, aliquots of approximately 5–10 μg of total DNA were digested with an appropriate restriction enzyme, separated by electrophoresis in 0.8% (w/v) agarose gels, and transferred onto Hybond-$N^+$ membranes. Southern hybridization was performed using the ECL Direct Nucleic Acid Labeling and Detection System (Cytiva) according to the manufacturer's instructions.

### 2.2 | Production of Fungal Inoculum for Infection

All strains of *T. benhamiae* (Table 1) were grown on SDA at 30°C, and spore suspensions were produced following a recently published method [26]. Briefly, after growth on SDA, dermatophyte sporulation was induced by incubation on potato dextrose agar at 30°C and under 12% of $CO_2$. The fungal material was then harvested, filtered through Miracloth layers (Millipore, Overijse, Belgium) and unicellular spores were suspended in PBS. The total concentration of spores in the resulting suspension was determined by counting colony forming units (CFU) after 3 days of incubation on SDA at 30°C.

To produce germ tubes and mycelium mixture, $1 \times 10^7$ CFU were grown in Sabouraud glucose broth for 24 h at 30°C under agitation (110 rpm). The germ tubes and mycelium were harvested on Miracloth layers and washed twice with 20 mL of PBS.

### 2.3 | Murine Infection Model

All animal experiments were conducted in compliance with European regulations. The protocols were approved by the Walloon authorities and the ethics committee of the University of Liège (agreement number: 22-2479).

Infection in the mouse model was performed as previously described [14]. Briefly, epicutaneous inoculation was performed in two steps: 100 mg of germ tubes and mycelium mixture were applied to 1 $cm^2$ of a scarified area on the occipital zone, and then 100 μL of a sterile 5% Poloxamer 407 (Merck, Darmstadt, Germany) solution containing $1 \times 10^8$ CFU was added on the top. Scarified but non-infected mice were used as negative controls.

### 2.4 | Clinical Score Analysis

After infection, the mice were monitored every 2 days by two independent examiners, who remained the same throughout the experiment, in double-blind conditions until the lesions had completely disappeared. Clinical scoring (/12) was performed, focusing on the intensity of three clinical signs: erythema (/4), scaling (/4) and crusting (/4). Skin biopsies were taken after Days 2 and 5 post-infection (PI) for histological analysis and total RNA extraction, as previously described [14].

### 2.5 | Histological Analysis

Biopsies taken from infected mice were fixed in a 4% formaldehyde solution and histologically processed as previously described [14]. Periodic-acid Schiff (PAS) staining with haematoxylin counterstaining was carried out to highlight fungal elements.

### 2.6 | RNA Extraction and RT-qPCR

Total RNA was extracted from mice skin biopsies and reverse transcribed into cDNA following previously optimised procedures [14]. After qPCR using Takyon NO ROX SYBR Master Mix (Eurogentec, Seraing, Belgium) and specific primers (listed in Tables S3 and S4), the relative mRNA expression of murine and fungal genes was calculated using the $2^{-\Delta\Delta Ct}$ method [27].

### 2.7 | Statistical Analyses

The G-Power3 software was used to determine the number of mice required for a meaningful subsequent interpretation of results. Using a one-sided proportion test with a risk of error of $\alpha = 5\%$ and a power of 95%, it was calculated that three mice per replicate and study day were necessary for clinical score analysis and two for histological and molecular analysis of the skin biopsies. All statistical analyses were performed using R software. Two-way ANOVA was carried out, and a $p \leq 0.05$ was taken as being significant.

## 3 | Results

### 3.1 | The *T. benhamiae* SUB6 Null Mutant Induces Superficial Skin Inflammatory Lesions in Mice Similar to Those Induced by the Parental Strain

The *T. benhamiae KU70*-lacking mutant (ΔKU70), derived from the wild-type (WT) strain, was used as the recipient strain to generate a *SUB6*-deleted strain and a *SUB6*-complemented strain as described in the materials and methods section. These latter two strains are referred to as ΔSUB6 and SUB6c strains throughout this article (Table 1). Mice were infected with each of these strains using a previously optimised procedure, resulting in significant and persistent lesions [14].

Mice were monitored every 2 days, and clinical scores were determined using three criteria: erythema, scaling and crusting. As there was a significant difference in clinical scores between male and female mice infected by *T. benhamiae* WT or ΔKU70 strains, with males exhibiting more severe lesions, all experiments conducted in this study were performed exclusively using males (Figure S2). Mice infected with the ΔKU70 strain showed less scaling and crusting than those infected with the WT strain, resulting in a slightly lower cumulative clinical score (Figure S3). To circumvent any problems in interpretation caused by this difference, mutant strains (ΔSUB6 and SUB6c) were compared exclusively with the *T. benhamiae* ΔKU70 parental strain.

Analysis of the clinical score curves of scarified but uninfected mice, used as negative controls (NC), showed that scarification induced lesions, but these were consistently lower than those of mice infected with the various strains studied, and statistical analysis confirmed that the lesions observed for the three clinical parameters were caused or aggravated by fungal infection (Figure 2A–D). Mice infected with the ΔKU70, ΔSUB6 or SUB6c strains showed similar clinical scores, demonstrating that the absence of the *SUB6* gene does not impair the development of lesions (Figure 2A–D). Tissue invasion by the *T. benhamiae* WT strain has previously been assessed over time by histological analysis, and Days 2 and 5 PI are key moments, corresponding to the onset of epidermal invasion and the peak of the fungal load within the tissues, respectively [14]. Similar observations were obtained for the ΔKU70, ΔSUB6 or SUB6c strains (Figure 3). Indeed, on Day 2 PI, numerous fungal spores and short filaments were observed

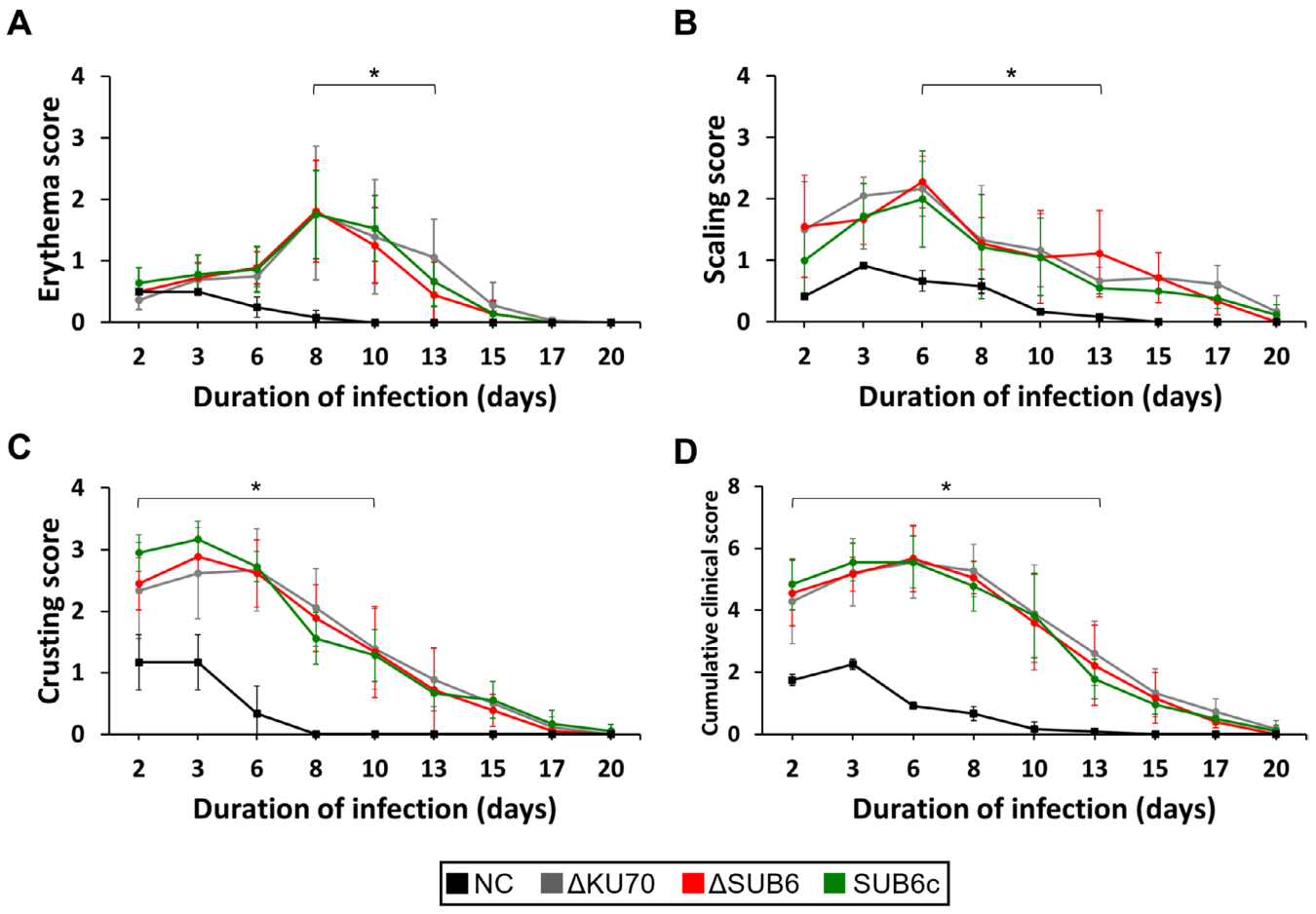


**FIGURE 2** | Clinical score with recipient (ΔKU70), *SUB6*-deleted (ΔSUB6) and *SUB6*-complemented (SUB6c) strains during infection in mice. Male mice were infected with recipient (ΔKU70), deleted for *SUB6* (ΔSUB6) and complemented for *SUB6* (SUB6c) strains of *T. benhamiae*. Lesions developed by infected mice were monitored until complete recovery with a clinical focus on the intensity of (A) erythema, (B) scaling and (C) crusting. (D) The global clinical score was derived from the cumulative scores of the previous three clinical signs ($n = 9 \pm$ SD; ANOVA2; $^*p < 0.05$; black asterisks indicate the statistical differences observed at different times during infection for the three clinical signs and the cumulative clinical score between each of the three strains compared with scarified but uninfected mice; NC, negative controls).

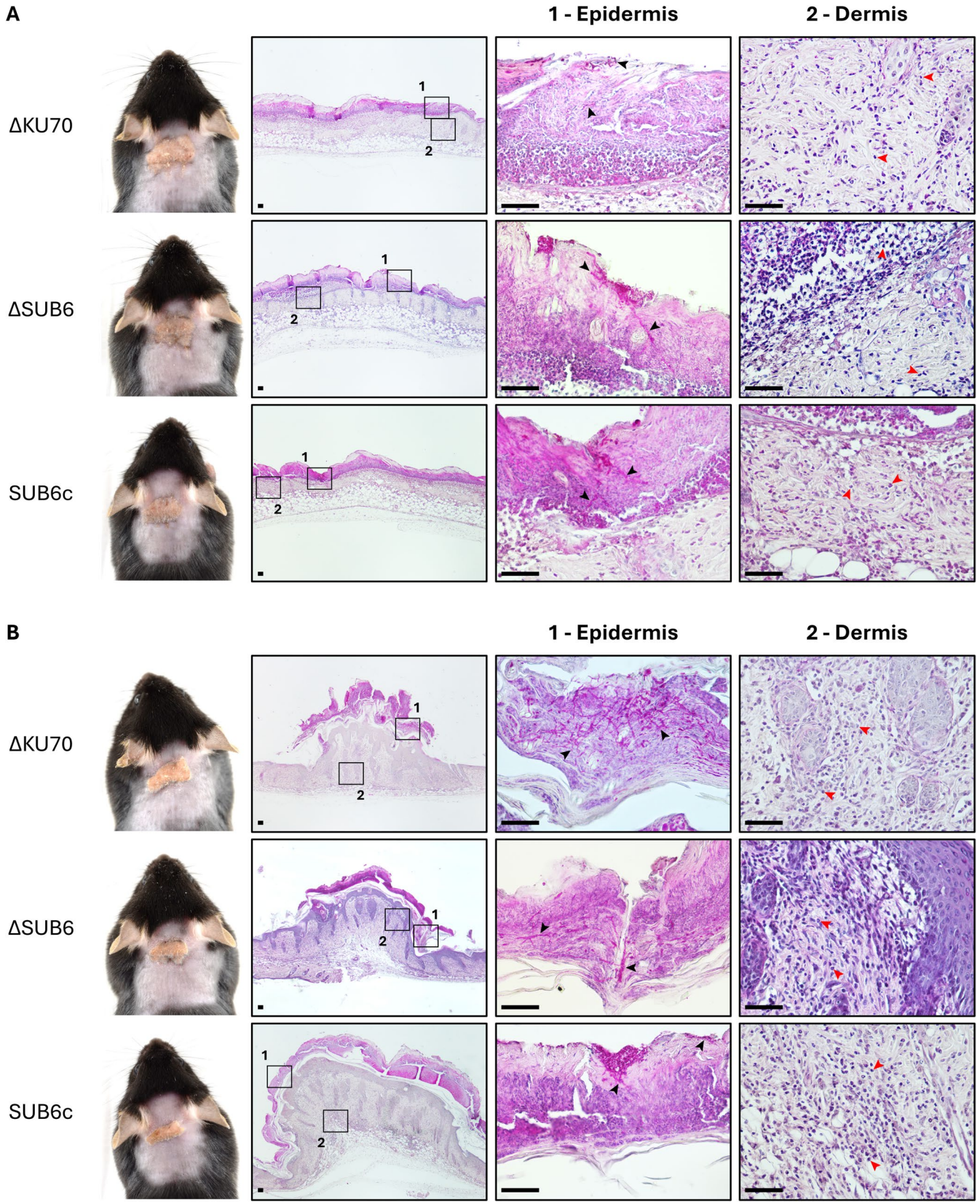


**FIGURE 3** | Fungal invasion and inflammatory cell infiltration of the ΔSUB6 and reference strains during infection in mice. Male mice were infected with recipient (ΔKU70), deleted for *SUB6* (ΔSUB6) and complemented for *SUB6* (SUB6c) strains of *T. benhamiae*. Skin biopsies were collected on (A) 2d and (B) 5d PI for histological processing and periodic acid-Schiff staining with haematoxylin counterstaining. The first histological column shows a general view of the infected area, pictures in the middle column show the epidermal zones where the fungal invasion is contained and the right column the dermis holding inflammatory infiltrate. The presence of fungi is indicated by black arrows ➤ and polymorphonuclear cells by red arrows ➤. Scale bar: 50 μm. 2d, Day 2; 5d, Day 5; PI, post-infection.

in the cornified layer as the crust began to form on the surface of the epidermis. On Day 5 PI, longer and more abundant fungal filaments were present in the superficial epidermis and in the remaining crusts. An intense inflammatory response, characterised by a dermal and perifollicular infiltrate of mononuclear and polymorphonuclear cells, was also observed concomitantly with fungal invasion of the tissues (Figure 3).

### 3.2 | Overexpression of Pro-Inflammatory Cytokines and β-Defensin-2 Genes Is Similar in Mice Infected With the *T. benhamiae* SUB6 Mutant and the Parental Strain

Besides histopathology, the host inflammatory response during infection with the different strains of *T. benhamiae* was also assessed by measuring the relative mRNA expression of three pro-inflammatory cytokines (*IL-1β*, *IL-12β* and *TNFα*) and one antimicrobial peptide (*β*-defensin 2, *BD2*) by RT-qPCR after extraction of total RNA (Figure 4; Figure S4). All of these inflammatory markers were significantly overexpressed on Day 2 PI in infected mice, whatever the strain used, but not on Day 5 PI, compared to the control condition corresponding to uninfected tissues.

### 3.3 | Genes of the Subtilisin Family Are Not Differently Expressed by the *T. benhamiae* SUB6 Null Mutant and the Parental Strain During Infection in the Mouse Model

Fungal subtilisin mRNA expression was assessed by RT-qPCR during the infection of mice with each of the *T. benhamiae* strains on Days 2 and 5 PI (Figure 5; Figure S5). Except for the *SUB8* gene, which was only overexpressed on Day 5 PI, the same expression profile was observed on Days 2 and 5 PI, with significant overexpression of the *SUB1*, *SUB3* and *SUB10* genes by all strains. Nevertheless, no differences were observed in the expression profile of subtilisin genes between the parental ΔKU70, ΔSUB6 and SUB6c strains.

## 4 | Discussion

A mouse model of *T. benhamiae* dermatophytosis was used to examine the contribution of the SUB6 protease to the virulence of the fungus. This model [14] was recently validated in terms of fungal gene expression during infection against a reference infection model in guinea pigs [13], which are the natural host of *T. benhamiae*. We took care to use a standardised inoculum to induce a natural-like superficial infection in mice. In this study, we successfully demonstrated transformation and disruption of the *SUB6* gene in the zoophilic dermatophyte *T. benhamiae*. However, significant differences in cutaneous clinical signs were observed between the WT and the ΔKU70 strains. Consequently, the phenotypic and functional results of the ΔSUB6 mutant were directly compared with those of the ΔKU70 strain. In addition, the deletion in the ΔSUB6 strain was compensated via in situ complementation, successfully integrating the gene into its native locus by homologous recombination, resulting in the SUB6c strain. The behaviour of the ΔSUB6 mutant was therefore finally compared with that of the ΔKU70 and the complemented SUB6c strains, hereafter referred to as reference strains.

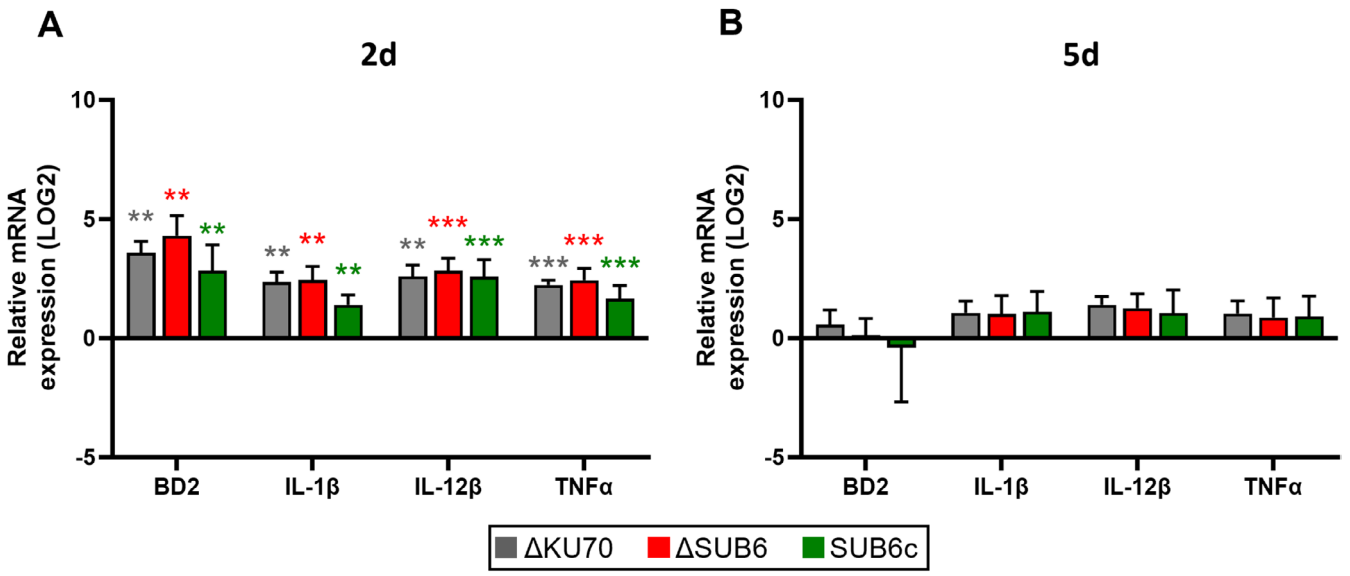


**FIGURE 4** | Host inflammatory responses of the ΔSUB6 and reference strains during infection in mice. Mice were infected with recipient (ΔKU70), deleted for *SUB6* (ΔSUB6) and complemented for *SUB6* (SUB6c) strains of *T. benhamiae*. The relative mRNA expression of murine pro-inflammatory cytokines and anti-microbial peptide was assessed by RT-qPCR after total RNA extraction from skin biopsies on (A) 2d and (B) 5d PI. The mRNA expression is relativized to that of the same gene measured in SNI mice ($n = 6 + SD$; ANOVA2; ** < 0.01, and *** $p < 0.001$; coloured asterisks show statistical differences for each strain compared to expression in control condition; no statistical differences are observed between strains $p > 0.05$). Complete names and accession numbers of studied genes are listed in Table S3. 2d, Day 2; 5d, Day 5; PI, post-infection; SNI, scarified but noninfected.

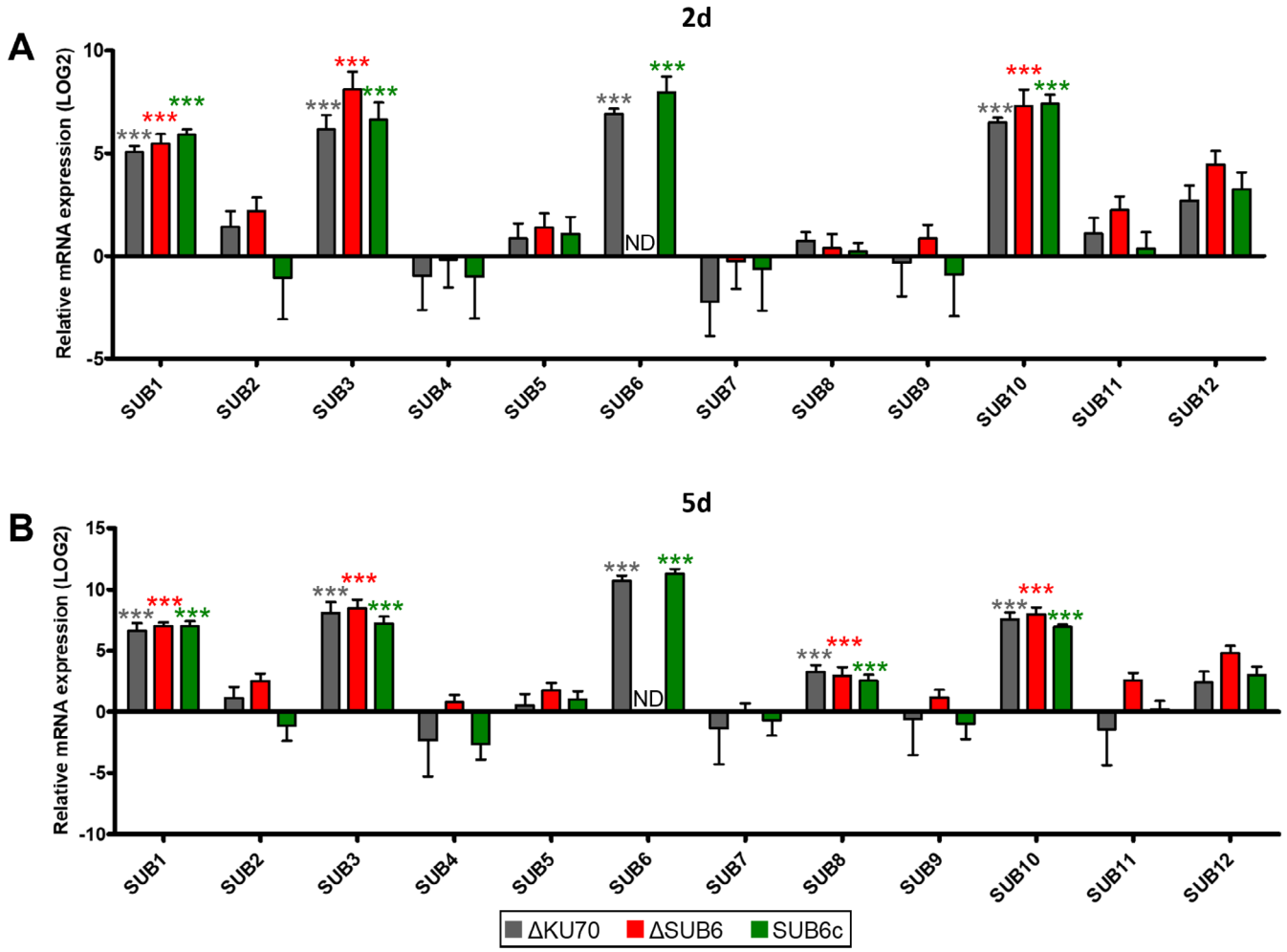


**FIGURE 5** | Fungal subtilisins expression in the ΔSUB6 and reference strains during infection in mice. Mice were infected with recipient (ΔKU70), deleted for *SUB6* (ΔSUB6) and complemented for *SUB6* (SUB6c) strains of *T. benhamiae*. The relative mRNA expression of fungal subtilisins was assessed by RT-qPCR after total RNA extraction from skin biopsies on (A) 2d and (B) 5d PI. The mRNA expression is relativized to the level of the same transcript measured in the fungal complex mixture before inoculation ($n = 6 + \text{SD}$; ANOVA2; $^{***}p < 0.001$; coloured asterisks show differences compared to control condition for each strain; ND, undetected Cq > 45). Complete names and accession numbers of studied genes are listed in Table S4. 2d, Day 2; 5d, Day 5; PI, post-infection; SUB, subtilisin.

### 4.1 | Comparable Clinical Signs and Histopathological Findings Between the SUB6 Null Mutant and Reference Strains

In our analysis of clinical scores and histological tissue invasion, no significant differences were observed between the ΔSUB6 mutant and the ΔKU70 strain of *T. benhamiae* in the mouse model. Both strains induced similar skin lesions, characterised by erythema, scaling and crusting, with comparable fungal loads in the infected epidermal layers. This suggests that the ΔSUB6 mutant retains the ability to colonise keratinised structures in a manner equivalent to the parental strain.

Our results contrast with those previously published demonstrating reduced virulence of the strain '*Trichophyton mentagrophytes*' ATCC 28185 with a deleted *SUB6* gene during dermatophytosis in a guinea pig model [28], bearing in mind that this '*Trichophyton mentagrophytes*' ATCC 28185 strain has recently been reclassified as *Trichophyton interdigitale*, which is an anthropophilic dermatophyte [29]. The observed reduction in clinical signs, considered in the light of the reassignment of strain ATCC 28185 to an anthropophilic species and together with our results, could suggest a fundamental difference in enzyme systems or degree of gene involvement during host infection between zoophilic and anthropophilic species, as was also recently demonstrated in vitro on RHE between *T. rubrum* and *T. benhamiae* [14].

### 4.2 | Comparable Variations in Pro-Inflammatory Gene Expression Between the SUB6 Null Mutant and Reference Strains

Invasion of the epidermis by fungal hyphae is accompanied by an inflammatory response in the host. The infection site is infiltrated by immune cells, mainly mononuclear and polymorphonuclear cells, in accordance with the role of macrophages in fungal clearance [5, 6, 30, 31]. Overexpression of the three pro-inflammatory cytokines (*IL-1β*, *IL-12β* and *TNFα*) and antimicrobial peptide *BD2* on Day 2 PI further corroborates that the Th1 and Th17 immune responses provide an effective response against dermatophytosis

[32–34]. However, no significant differences were observed in the expression of inflammatory markers between the ΔSUB6 and ΔKU70 strains. This contrasts with the results reported for the ΔSUB6 deleted strain of *T. interdigitale* ATCC 28185, which exhibited reduced production of IFNγ, IL-12β and TNFα during infection compared with the WT strain [28]. The observed difference in behaviour highlights the complexity of host-pathogen interactions and the importance of biological context in the study of fungal virulence. *Trichophyton interdigitale* might induce more pronounced immune modulation, while *T. benhamiae* might activate alternative inflammatory pathways or not rely as heavily on the *SUB6* gene to modulate the host immune response [28].

### 4.3 | Complex Interplay of Fungal Virulence Factors: Potential Host- and Species-Specific Mechanisms in Dermatophytes

During skin invasion in mice, other *T. benhamiae* genes were specifically overexpressed during infection, in particular genes encoding proteins such as a naphthalene reductase (ARP2) and a deuterolysin (DEUT) [14]. All the proteins encoded by these genes represent interesting markers of dermatophytosis, potentially involved in the virulence of the fungus. In addition, other genes encoding subtilisins such as *SUB1*, *SUB2*, *SUB3*, *SUB4*, *SUB7* and *SUB8* were differentially expressed depending on the model used (mouse, guinea pig or RHE), but also on the dermatophyte species, suggesting once again the existence of host- and species-specific pathogenic mechanisms in dermatophytes [13, 14]. The expression profiles of the 12 *SUB* genes were analysed to gain deeper insights into the mechanisms underlying dermatophyte virulence and to explore potential compensatory or inhibitory interactions between subtilisins during infection. The ΔSUB6 strain of *T. benhamiae* showed significant overexpression of the *SUB1*, *SUB3*, *SUB8* and *SUB10* genes, similar to what was observed for the reference strains, suggesting the absence of compensatory interactions between subtilisins. Compensatory or inhibitory mechanisms between dermatophyte proteases have previously been reported, such as the upregulation of *MEP4* and *SUB3* genes alongside a marked downregulation of *MEP1*, *MEP2* and *MEP5* genes in the ΔSUB6 strain of *T. interdigitale*, but these results were only obtained in vitro [28], which does not necessarily reflect in vivo pathogenic mechanisms.

In conclusion, the comparable virulence observed in the ΔKU70 and ΔSUB6 mutants of *T. benhamiae* suggests that the *SUB6* gene is neither essential for pathogenicity nor for growth of the fungus in the in vivo mouse model of dermatophytosis used here. To date, no study has comprehensively investigated the role of subtilisins in *T. benhamiae* through gene deletion. The closest study is by Grumbt and colleagues, who addressed the role of a malate synthase by site-directed mutagenesis using the *T. benhamiae* strain IHEM 20163 in a guinea pig model of infection [21]. Their results also showed similar fungal development and tissue invasion between strains and suggest that inflammatory symptoms in zoophilic dermatophyte infections are more closely tied to host responses. Disrupting a single gene in this subtilisin family may not fully abolish pathogenicity [9, 35–37]. It therefore seems essential to investigate the behaviour of multiple mutants across different infection models and in dermatophyte species from distinct ecological niches.

#### Author Contributions

**Wilfried Poirier:** conceptualization, methodology, data curation, investigation, validation, formal analysis, supervision, visualization, project administration, writing – original draft, writing – review and editing. **Émilie Faway:** conceptualization, funding acquisition, methodology, project administration, supervision, validation, visualization, writing – original draft. **Tsuyoshi Yamada:** conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, writing – original draft. **Kiyotaka Ozawa:** investigation. **Françoise Maréchal:** investigation. **Michel Monod:** conceptualization, methodology, supervision, validation, writing – original draft, writing – review and editing. **Yves Poumay:** conceptualization, funding acquisition, methodology, supervision, validation, writing – original draft. **Bernard Mignon:** conceptualization, funding acquisition, methodology, supervision, validation, writing – original draft, writing – review and editing.

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#### Conflicts of Interest

The authors declare no conflicts of interest.

#### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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