A relevant new mouse model of superficial dermatophytosis reveals that subtilisin 6 of *Trichophyton benhamiae* is not essential for virulence

W. Poirier[1], E. Faway[2], F. Maréchal[1], J. Piret[1], Y. Poumay[2], B. Mignon[1]

[1]*Faculty of Veterinary Medicine, University of Liège (Belgium)*

[2]*Namur University ASBL (Belgium)*

Dermatophytoses are superficial cutaneous mycoses observed in both humans and animals caused by filamentous fungi called dermatophytes. Human dermatophytosis is widespread and most often caused by species belonging to the genus *Trichophyton*. The increasing emergence of strains resistant to currently available antifungals requires the development of new treatments. In this context, a better knowledge of the pathogenesis of dermatophytosis, including the identification of fungal virulence factors such as subtilisins, is a key step for the identification of new therapeutic targets. Our aims were (1) to develop a robust and relevant mouse model of superficial dermatophytosis to study host-pathogen interactions, using *Trichophyton benhamiae* as a reference species, and (2) to assess the role of a secreted protease, subtilisin 6 (SUB6), in virulence.

Optimization of the epicutaneous infection model using *T. benhamiae* IHEM 20161 wild-type

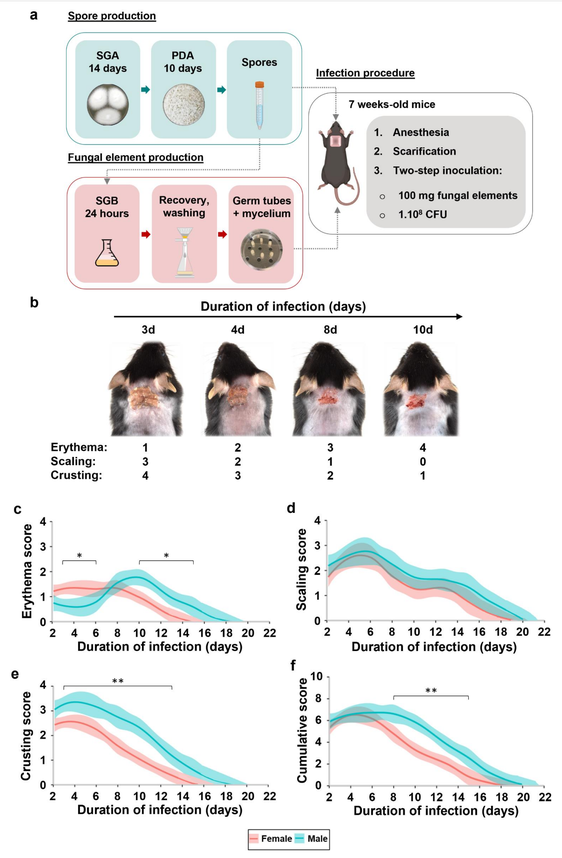

Figure 1: Development of the infection by *T. benhamiae* in mice. (**A**) New method of mice infection. (**B**) Based on three clinical parameters, (**C-F**) lesions developed by infected mice were monitored until complete recovery and clinical scores were assigned (n=9±SD; ANOVA2; *p<0.05, **p<0.01). 10d, day 10; 3d, day 3; 4d, day 4; 8d, day 8; CFU, colony-forming unit; PDA, potato dextrose agar; SGA, Sabouraud glucose agar; SGB, Sabouraud glucose broth.

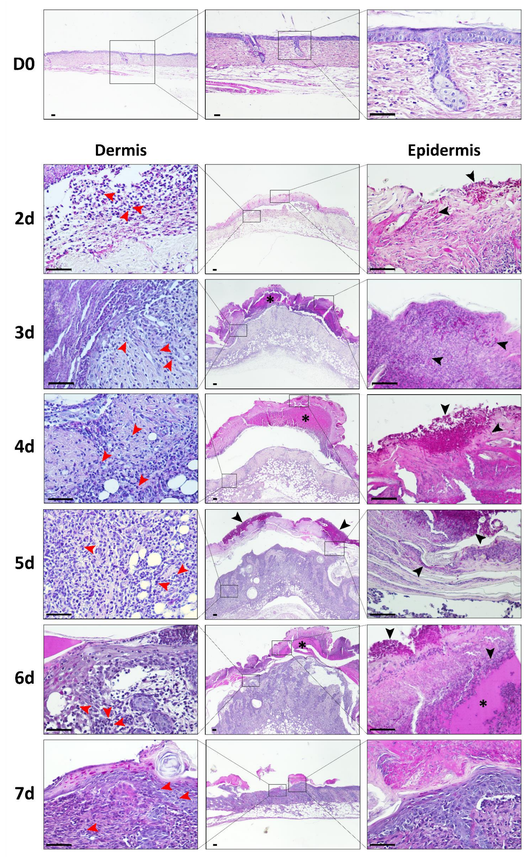

Figure 2: Fungal invasion and inflammatory cell infiltration during *T. benhamiae* infection in mice. Biopsies were recovered from lesional skin daily from days 2 (2d) to 7 (7d) PI, then histologically processed and stained by periodic acid-Schiff. The presence of dry pustules within the infected tissue is indicated by asterisks, fungi by black arrows (➤), and neutrophils by red arrows (➤). Scale bars: 50 µm.

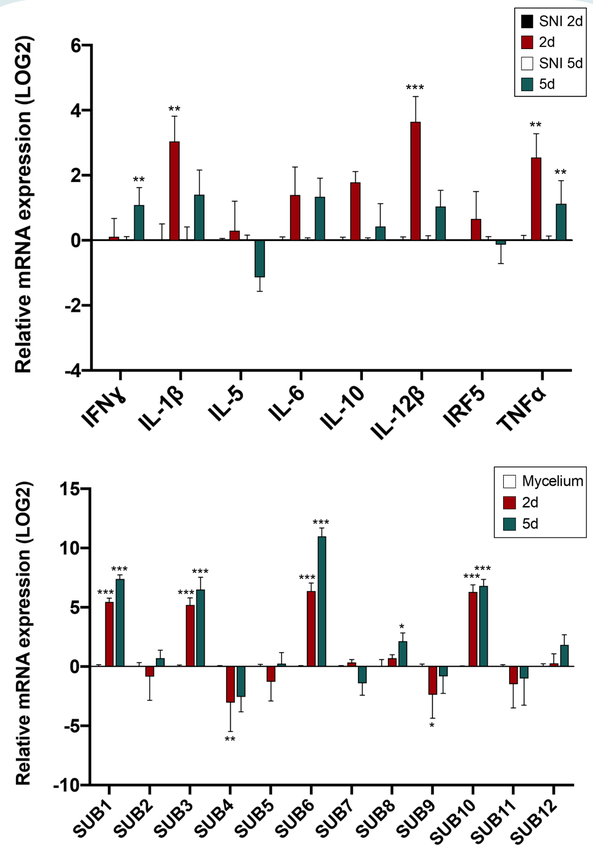

Figure 3: Host inflammatory responses and fungal subtilisins expression during *T. benhamiae* infection in mice. The relative mRNA expression was assessed by RT-qPCR after total RNA extraction from skin biopsies on days 2 (2d) and 5 (5d) PI. The mRNA expression is relativized to the expression of the same gene measured in scarified but non-infected mice (SNI) or in the mycelium (n=6+SD; ANOVA2; *p<0.05, **p<0.01, ***p<0.001).

Role of subtilisin 6 (SUB6) in the virulence of *T. benhamiae* IHEM 20161

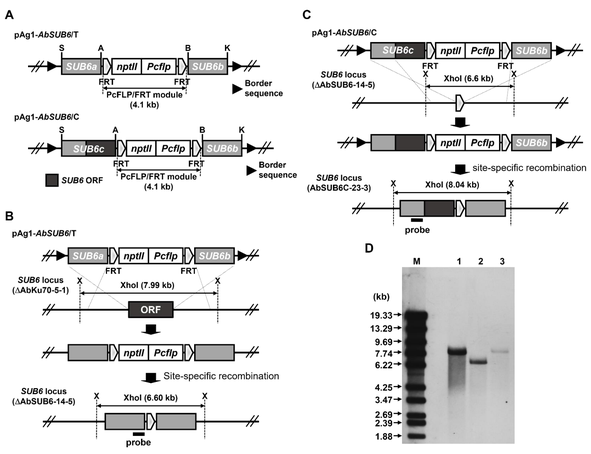

Figure 4: Disruption of the *SUB6* gene of *T. benhamiae* ΔKU70 and subsequent reintroduction of the *SUB6* gene by a gene replacement strategy.

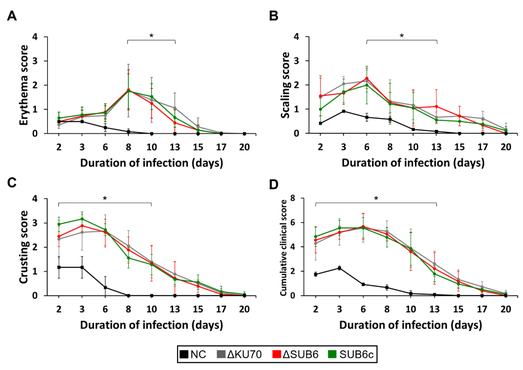

Figure 5: Clinical score with recipient (ΔKU70), SUB6-deleted (ΔSUB6) and SUB6-complemented (SUB6c) strains during infection in mice. Lesions developed by infected mice were monitored until complete recovery with a clinical focus on the intensity of (**A**) erythema, (**B**) scaling and (**C**) crusting. (**D**) The global clinical score was derived from the cumulative scores of the previous three clinical signs (n=9±SD; ANOVA2; *p<0.05).

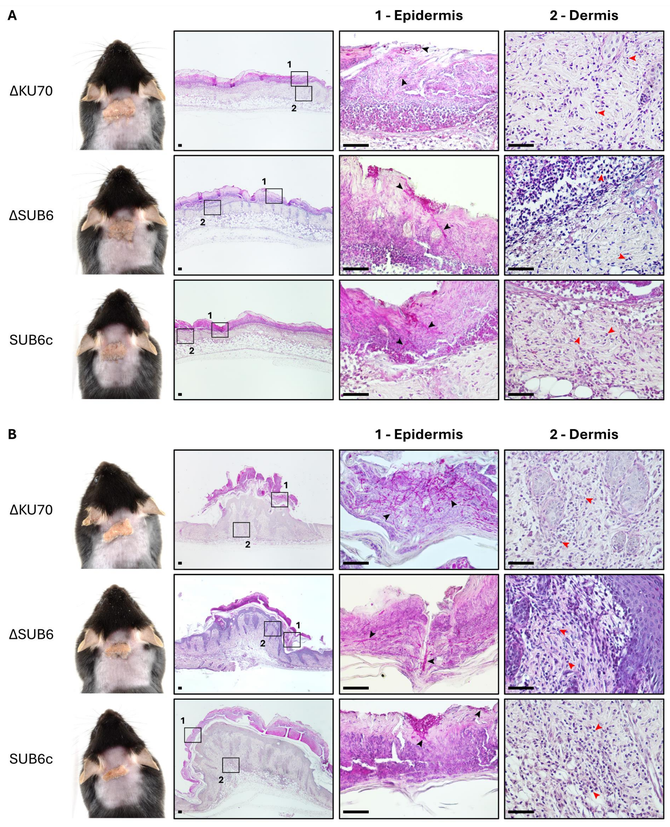

Figure 6: Fungal invasion and inflammatory cell infiltration of the ΔSUB6 and reference strains during infection in mice. Skin biopsies were collected at days (**A**) 2 and (**B**) 5 PI for histological processing and periodic acid-Schiff staining with hematoxylin counterstaining. For each time point, the left column shows 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.

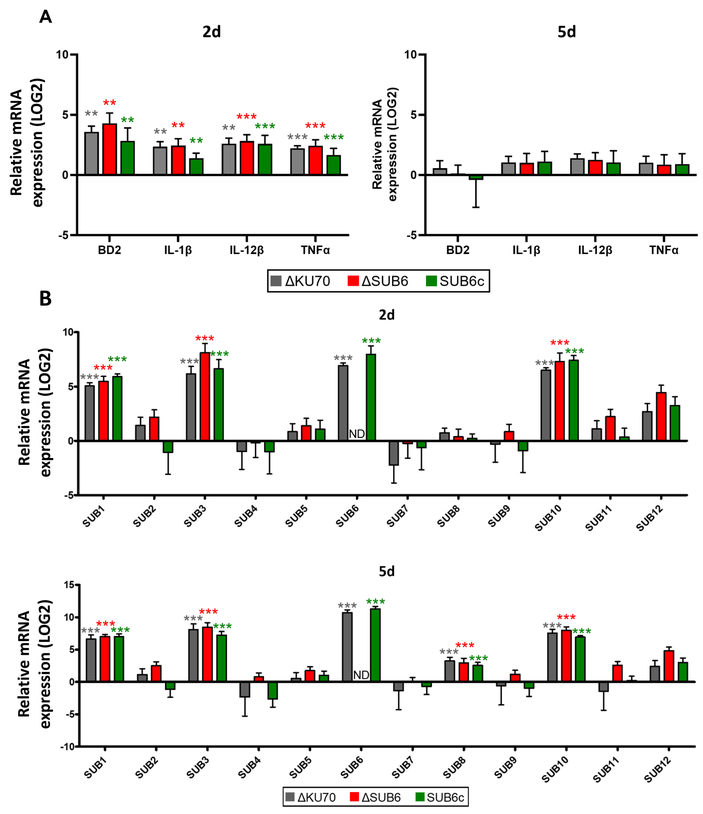

Figure 7: Host inflammatory responses (A) and fungal subtilisins (B) expression in the ΔSUB6 and reference strains during infection in mice. The relative mRNA expression was assessed by RT-qPCR after total RNA extraction from skin biopsies on days 2 (2d) and 5 (5d) PI. The mRNA expression is relativized to the expression of the same gene measured in scarified but non-infected mice (SNI) or in the mycelium (n=6+SD; ANOVA2; *p<0.05, **p<0.01, ***p<0.001; ND, undetected Cq > 45).

The specific, standardized inoculum prepared from the wild-type strain of *T. benhamiae*, generated visible cutaneous symptoms mimicking a natural, self-resolving infection, lasting more than twice as long (16 days vs 7 days) as those obtained in the past with an inoculum containing only spores. Histopathological lesions showed a superficial invasion of the epidermis by hyphae and inflammatory infiltration of the epidermis and dermis by mononuclear and polymorphonuclear cells. While the pro-inflammatory cytokines *IL-1β*, *IL-12β* and *TNFα* were significantly overexpressed on day 2 after infection, the fungal genes *SUB1*, *SUB3*, *SUB6*, *SUB8* and *SUB10* were also strongly overexpressed during infection in both times. The ΔSUB6 strain induced superficial skin symptoms and histopathological inflammatory lesions similar to those caused by the parental strain. It also induced significant overexpression of the same host pro-inflammatory genes and fungal genes in the tissues, with no difference from the parental strain. Our new dermatophytosis mouse model is a powerful tool for studying the pathophysiology of acute superficial dermatophytosis. It has enabled us to show that certain subtilisins, including *SUB6*, are overexpressed during infection and could play an important role in the infectious process. However, SUB6, while being a marker of infection, is not a virulence factor, at least not acting alone.