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Can new probiont isolate of bovine milk reverse aflatoxin M1-induced neutrophil oscillation?

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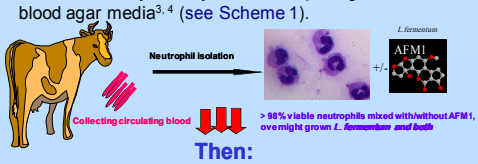
Introduction

In high yielding dairy cow infection is a big problem, and protection of animals from pathogens is a tough challenge for the bovine and dairy industries and public health. As a metabolite of aflatoxin (AF) B1, AFM1 is a potential granulotoxin, and in AFB1-exposed cows the metabolite appears everywhere, especially in mammary gland. Neutrophils are pivotal for defense against mastitis; they have enormous potentials to finally eliminate engulfed pathogens. We have recently observed the antagonistic properties of a *Lactobacillus* (*L.*) *fermentum*, isolated from milk of healthy dairy cows, against *Staphylococcus aureus* (*S. aureus*). The main purposes of this study were 1) to assess the effect of AFM1 on neutrophils killing activity against S. aureus in healthy dairy cows, and 2) to determine whether *L. fermentum* reverses the diminished effects of the AFM1 on neutrophils in healthy dairy cows.

Materials and Methods

Cows, blood samples and analyses: Healthy mid-lactating dairy cows (n=8) were used as a source of neutrophils; blood samples of the cows were aseptically collected for neutrophil isolation and neutrophil functional analyses[2, 3]. The isolated blood neutrophils were exposed with: 1) only 25 ng/ml of AFM1 2) AFM1 plus overnight grown *L. fermentum* 3) only overnight grown *L. fermentum* and 4) none of them for 3 hours; their capacity to kill *S. aureus* was then monitored by a bactericidal assay, using *in vitro* challenge of *S. aureus,* Newbould 305, which are routinely isolated from clinical cases of mastitis, with above mentioned neutrophil groups for 1 h and then cfu counting of the *S. aureus* was monitored by a bactericidal assay, accordingly[3].

Bactericidal assays: Briefly, 100 µl live *S. aureus* (5×10^7 / ml) were added to 500 µl viable neutrophils (5×10^6 / ml) already exposed with/without AFM1, *L. fermentum,* AFM1 plus *L. fermentum*, or to 500 µl of normal saline containing no neutrophils, incubated for 1 hour, and finally serially diluted for plating out on the blood agar media[3, 4] (see Scheme 1).



Scheme 1. Blood neutrophil isolation and phagocytosis and killing assay with / without AFM1 and *L. fermentum*.

To see the effect of four above mentioned treatments on phagocytosis and killing activities on the *S. aureus,* after dilution and plating out, they were mixed with a sterile plastic loop in triplicates onto Columbia sheep blood agar (Biokar Diagnostic, Beauvois, France) in 5% CO_2 at 37°C for 24 h, and colony counts were performed. The log CFU/ml of each sample was calculated, and results from the bacteriological assay are expressed and compared as the percentage of killed (% killing) of *S. aureus*[3, 4] (see the formula in the Scheme 1).

Results and discussion

The bactericidal activity of neutrophils against *S. aureus* in AFM1-treated group was ~23 %, and ~43 % in control group. Similar pattern was observed on phagocytosis activity, that is, the phagocytosis of *S. aureus* by AFM1-exposed neutrophils significantly diminished ($P < 0.01$; figures 1 and 2).

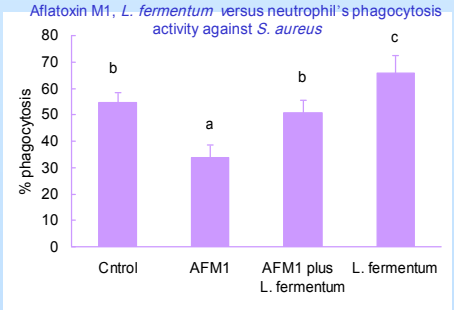


Figure 1. Effect of AFM1, *L. fermentum* and AFM1-*L. fermentum* co-culture on the phagocytosis activity of neutrophils against *S. aureus*. The neutrophils were incubated in four different treatment conditions for 3 hours. We can see a strong impairment of phagocytosis capacity of neutrophils against *S. aureus* by AFM1, but reverse results from *L. fermentum* and/or combination of AFM1 and *L. fermentum* (n = 8 cows; values are means ± SEM; different superscript letters in the groups represent $p< 0.05$); all samples were measured in triplicates. For detailed information please see the methodology of the study.

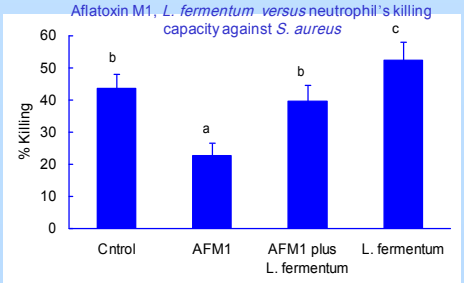


Figure 2. Effect of AFM1, *L. fermentum* and AFM1-*L. fermentum* co-culture on the killing capacity of neutrophils against *S. aureus*. The neutrophils were incubated in four different treatment conditions for 3 hours. As we can see a strong impairment of neutrophils' killing capacity against *S. aureus* by AFM1, but reverse results from *L. fermentum* and/or combination of AFM1 and *L. fermentum* (n = 8 cows; values are means ± SEM; different superscript letters in the groups represent $p< 0.05$); all samples were measured in triplicates. For detailed information please see the methodology of the study.

The phagocytosis capacity of neutrophils against *S. aureus* in AFM1-treated group was significantly lower compared to other groups. Interestingly, this adverse effects of AFM1 on neutrophils' engulfing and killing capacities was reversed when co-cultured with *L. fermentum* (see figures 1 and 2). Similar pattern was observed on phagocytosis activity of neutrophils when they pre-exposed with *L. fermentum*.

Further, both the killing activity and phogocytosis activity of neutrophils against this pathogenic superbug, *S. aureus,* were remarkably improved with the application of *L. fermentum* (see figures 1 and 2). The inhibiting the adverse effects of AFM1 on neutrophils' killing capacity with pre-exposing of neutrophils with the *L. fermentum* and/or co-culturing with *L. fermentum* is interestingly encouraging.
Our observation confirmed the fact that AFM1 is immunotoxic or antiphagocytic in dairy cows, especially affecting on the pivotally cellular part of circulating innate defence system.

The killing of S. aureus by AFM1-treated neutrophils was markedly minimal and improved significantly with application of the *L. fermentum*. Observed improved phagocytic activity of neutrophils sheds fresh light on the application of this lactobacillus as a good probiotic in farm animals to prevent infection especially in immunocompromised periparturient dairy cows.

Since neutrophils' oscillation happens in dairy cows around peripartum period, and AFM1 accelerates oscillatory events, finding potential immunostimuli is essential and *L. fermentum* can be considered as a good candidate for that purpose.
Further studies are needed to explain the cellular and molecular mechanisms happening in AFM1-*L. fermentum* – neutrophils interactions.

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