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Lactobacillus infection related to midgut protein synthesis in the dengue vector *Aedes albopictus*: Platform of non-symbiont bacteria for the control of *Aedes* vectors

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There have been a number of recent studies regarding the use of engineered insect symbiont bacteria for control of insect-borne diseases. However, searches for cultivable bacteria residing in the mosquito midgut have met with little success. The present study was conducted to evaluate the effects of the human non-pathogenic *lactobacilli* on midgut protein synthesis in the dengue vector *Aedes albopictus*, taking into account the ease of infection and its persistence. It was showed that antibiotic treatment of mosquitoes did not prevent experimental infection, and readily reduced undesired infection, but did not prevent re-infection by *Lactobacillus* spp. It suggests a high potential of colonization of a target vector population under field conditions. Ingested lactobacilli remained in the female midgut for five days. *Lactobacillus reuteri* (Lactobacillales: Lactobacillaceae) showed more specific proteins than *Lactobacillus brevis* (Lactobacillales: Lactobacillaceae). Some proteins identified in *L. brevis* were present at much higher levels in *L. reuteri*, while other proteins found in the latter were found at higher levels in the former. Infection by *L. brevis* resulted in the absence of many proteins. In contrast, *L. reuteri* infection resulted in increased levels of synthesis of a set of proteins present in the healthy midguts. Both bacteria triggered changes in midgut protein synthesis, but activation was seen to a greater extent with *L. reuteri*. These results are discussed in the context of paratransgenesis.

Key words: Bacteria, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Aedes albopictus*, midgut, ease of infection, persistence, protein synthesis.

INTRODUCTION

Vaccine development and insecticides used to be the main strategies for controlling mosquito-borne diseases, but the genetic variability of the pathogens and insecticide resistance have increasingly limited the success of such measures (Caetano and Yoneyama, 2001; Dieng et al., 2007). Another promising method, which consists of replacing wild populations with vector-incompetent transgenic mosquitoes, has been suggested. While the ability to generate refractory vectors is available (Jasinskiene et al., 1998; Catteruccia et al., 2000), a mechanism for replacing a wild vector population with a vector-incompetent refractory population remains elusive (Riehle and Jacobs-Lorena 2005). In addition, there are still no suitable gene carriers available for the transformation of some important vectors.

These include *Aedes albopictus*, a species originating in Southeast Asia, found on all continents and reported in

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25 countries (Juliano and Lounibos, 2005; Eritja et al., 2005). This mosquito is an important vector of yellow fever, diverse encephalitis, and several arboviruses, in particular dengue (Mitchell, 1995), which is one of the most important mosquito-borne diseases (Hales et al. 2002). Thus, the rapid dispersal and establishment of this species has serious public health implications, and there is obviously concern regarding the possibility of large-scale dengue outbreaks.

Recently, there has been a great deal of research regarding insect gut microbiota due to its status as a potential source of immune compounds (Chernysh et al., 2002). Furthermore, manipulation of these bacterial symbionts has been proposed as a strategy for insect-borne disease control (Lehane et al., 1997; Beard et al., 2002; Dillon et al., 2005; Favia et al., 2008). This approach, known as paratransgenesis, involves the bacterial delivery of effector molecules capable of inhibiting pathogen development (Beard et al., 2002; Lindh et al., 2006). Once established, bacteria induce an immune response and some can counteract the host defenses (Hoffman et al., 1996; Vallet-Gely et al., 2008). The degrees to which gene expressions are induced and host defenses are overcome vary among bacterial species. Thus, the assessment of induced gene expression may aid in evaluating bacteria as paratransgenic tools.

Almost all studies on bacteria relative to their use in paratransgenesis have used symbionts and their immune effects for analysis (Lindh et al., 2006; Favia et al., 2008; Durvasula et al., 1997). However, this may not be applicable to mosquitoes. Isolation of bacteria from mosquitoes has also been reported (Pumpuni et al., 1996; Khampang et al., 1999; Moll et al., 2001; Pidiyar et al., 2004; Gonzalez-Ceron et al., 2003). Many species have no cultivable bacteria in their midgut (Gusmão et al., 2007); those that do generally harbor only a single species (Riehle and Jacobs-Lorena, 2005).

Most studies to identify candidate paratransgenic bacteria did not address the likely possibility that insect non-symbiotic bacteria may also be valuable candidates. In fact, once a key bacterium is found, it may be genetically modified to affect pathogen development, and consequently reduce disease transmission (Azambuja et al., 2005; Riehle and Jacobs-Lorena, 2005). Such modifications may result in a bacterium that is different from the original and may not have the same interactions with the insect in which it resided. Therefore, any bacterium that is not a symbiont, but has high cultivability and genetic transformability, could also be valuable candidates for paratransgenesis. For such a novel para-transgenic approach based on non-symbiotic bacteria, the ease of infection by the candidate is critical. The present study was conducted to evaluate the effects of the human non-pathogenic lactobacilli on midgut proteins synthesis in the dengue vector *A. albopictus*, taking into account the ease of infection and its persistence.

MATERIALS AND METHODS

Mosquito

A. albopictus used here originated from a colony maintained under conditions of 26°C, 60 - 80% RH, and 12:8 h photoperiod. Routinely, larvae were supplied 3 g of a mixture of powdered mouse pellet diet (CLEA Japan, Inc., Tokyo, Japan) and dry yeast (Tanabe Seiyaku Co. Ltd., Osaka, Japan) (1:1) three times during development. Adults were fed a 10% sucrose solution and females were blood-fed on immobilized mice. Adults used in this study were maintained on a 10% sucrose diet and did not have access to blood.

Bacteria

Three non-pathogenic bacterial strains, *Lactobacillus casei*, *L. brevis* and *L. reuteri*, were grown in Man-Rogosa-Sharpe liquid medium (MRS) at 37°C for 14 h. The numbers of bacterial cells were determined by measuring the optical density at 660 nm (OD_{660}) using a NovaSpec II spectrophotometer (LKB Biochrom, Cambridge, UK). Aliquots of 5 ml of bacterial cultures were centrifuged at 5000 rpm for 10 min at 4°C. Pellets were washed with sterilized phosphate saline buffer (PBS) and resuspended in sterilized 10% sucrose solution. Mosquitoes were fed sugared bacterial suspensions of approximately 4.0×10^7 colony forming units (cfu) per ml.

Antibiotic treatment and experimental feeding

It was first examined whether antibiotic treatment can yield mosquitoes free of bacteria. For this, a group of approximately 100 females fed a diet of 10% sucrose were starved for 12 h and then divided into two groups, which were subjected to one of the following regimes for 3 days: (1) 10% sucrose with 15 µg of erythromycin per ml (treated) or (2) the same meal but without the antibiotic (untreated). Subsequently, 8 individuals from each group were sampled and examined for bacterial infection. Treated mosquitoes were further divided into two sub-groups and allowed to feed for 3 days on one of the following meals: (i) 10% sucrose solution mixed with ~4.0×10^7 cfu per ml of *L. casei* or (ii) 10% sucrose solution. The following experiment was performed based on the results presented in Table 1. One hundred erythromycin-treated female *A. albopictus* mosquitoes were starved as described previously and then allowed to feed over a period of 3 days on one of the following diets: (a) 10% sucrose solution mixed with ~4.0×10^7 cfu per ml of *L. brevis* or (b) 10% sucrose solution mixed with ~4.0×10^7 cfu per ml of *L. reuteri*. Physiologically similar females that had access to 10% sucrose solution but were not treated with erythromycin were used as controls.

Dissection and detection of bacterial infection

The midguts were dissected from females untreated with erythro-mycin and fed 10% sucrose, erythromycin-treated females fed 10% sucrose solution infected with *L. casei*, erythromycin-treated females fed 10% sucrose solution infected with *L. brevis* and erythromycin-treated females fed 10% sucrose solution infected with *L. reuteri*. All dissections were performed aseptically in droplets of cool sterilized Phosphate Buffered Saline (PBS). Dissection materials were wiped frequently with isopropanol and slides, forceps, and pins were changed after each dissection. Dissected undamaged midguts were incised, washed with PBS and stored at -80°C. The presence of a bacterial population in mosquitoes was

Table 1. Prevalence of bacterial infection in the midgut of female *A. albopictus* mosquitoes treated with erythromycin and after infection with *L. casei*.

	No. females tested	No. midgets with *Lactobacillus sp*		% mosquitoes infected with *Lactobacillus sp*	
		2 days	5 days	2 days	5 days
Erythromycin-treated females	8	2	1	25	12.5
Untreated females	8	4	-	50	-
L. casei infection after antibiotic treatment					
Erythromycin-treated/*L. casei* infected sugar-fed females	8	5	8	62.5	100
Erythromycin-treated/10% sugar-fed females	8	1	1	12.5	12.5

assessed in duplicate by culture of homogenates in MRS medium. Aliquots of 100 µl of midgut homogenate were spread on MRS agar plates, which were then incubated at 37°C for 48 h.

Protein extraction, concentration and electrophoresis

Proteins were extracted from the midguts of mosquitoes and from bacteria. Twenty-four pooled midguts and post-exponential phase bacterial cultures were homogenized in pre-cooled lysis buffer (PRO-PREP™ Protein Extraction Kit; iNtRON Biotechnology, Seoul, Korea). Homogenates were centrifuged for 10 min at 4°C and 13,000 rpm for midguts and at 5000 rpm for bacteria. Supernatants were then transferred to new tubes and stored at -20°C for further analysis. Aliquots of the supernatants were used to determine protein quantities in triplicate using a modification of the Bradford method (Bradford, 1976) with an ImmunoMini NJ-2300 immunoreader (Nippon InterMed, Tokyo, Japan) set to monitor absorbance at 590 nm. Concentrations are expressed as optical densities. SDS-polyacrylamide gel electrophoresis was performed using midgut homogenates from non-infected, *L. brevis*-infected meal-fed females, *L. reuteri*-infected meal-fed females, and extracts from *L. brevis* and *L. reuteri* cultures. Aliquots of 1.5 µg of protein from each sample were run on the gels and the molecular weights of proteins in the gels were estimated by applying 2.5 µl of Precision Plus Proteins™ markers (Bio-Rad, Hercules, CA) to each gel run. Electrophoresis conditions were 200 mA, 400 volts for 90 min, and proteins were visualized by silver staining.

Data collection and analysis

The efficacy of antibiotic treatment was calculated as the number of antibiotic-treated mosquitoes with no infection divided by the number of tested for Lactobacilli infection. The silver-stained gels were scanned (GT-7600U; Epson, Singapore) and transferred into Adobe Photoshop Software (Adobe Systems Inc., San Jose, CA). Band patterns were analyzed visually and assigned according to Prévot et al. (2003). The effects of infection on protein synthesis were assessed by comparing protein profiles from non-infected and bacteria-infected midguts. Two gels were used for these comparisons: (i) 13% polyacrylamide gels for comparing proteins of high molecular weight (200-25 kDa) between the midguts of *L. reuteri*-infected and non-infected mosquitoes, and (ii) between the midguts of *L. brevis*-infected and non-infected mosquitoes; and 16% polyacrylamide gels for comparing proteins of low molecular weight (25 - 10 kDa) between the midguts of non-infected, *L. brevis*-infected, and *L. reuteri*-infected mosquitoes. Due to a high number of bands, (a) the comparison of protein profiles between *L. brevis* and *L. reuteri* was based on the detection of at least 20 shared bands and at least 4 specific ones; (b) the comparison of the synthesis profiles of high molecular weight proteins (200 - 25 kDa) between infected and non-infected midguts was based on the detection of at least 12 shared bands; (c) the comparison of the synthesis profiles of low molecular weight proteins (25 - 10 kDa) between infected and non-infected midguts was based on the detection of at least 8 shared bands. Band patterns were eye-analyzed as to follow Dieng et al. (2007) and discrepancies considered if observed in at least two of the four replicates. In all analyses, only differences related to the differences in darkness/lightness between identical bands were taken into account. The stronger of two identical bands had a higher expression level.

RESULTS

Efficacy of erythromycin treatment and infectivity post-antibiotic treatment

Treatment with 15 µg of erythromycin resulted in a *Lactobacillus* sp prevalence rates of 12.5 and 50% in the midguts from *A. albopictus* in the treated and non-treated groups, respectively. This indicates the ease with which *Lactobacillus* infects. Three days post-antibiotic treatment, all females tested that had been fed *L. casei*-infected meal showed infection by the bacteria. Five days after feeding on the infected meal, all erythromycin-treated *L. casei* infected sugar-fed females were still harboring the bacteria (Table 1).

Midgut protein content

Protein quantities were determined in triplicate for each of the bacterium species and for each of the midgut physiological status. In both cases, protein concentrations were variables (Table 2). We calculated mean protein concentrations for infected midguts and observed that the protein content of midguts from healthy females did not significantly change following the uptake of a bacteria-infected meal (Table 3).

Bacterial proteome

The comparison of the protein synthesis profiles between

Table 2. Protein contents in the midgut of female *A. albopictus* mosquitoes.

	Mean concentration ± SE (µg/ml)
L. brevis-infected meal-fed midgut	170.28 ± 17.2
L. reuteri-infected meal-fed midgut	58.86 ± 2.75
Sugar fed midgut	109.43 ± 7.92
L. brevis (57.6×10^8 cfu/ml)	115.86 ± 2.28
L. reuteri (90.53×10^8 cfu/ml)	143.23 ± 5.44

Table 3. Concentration of proteins in *A. albopictus* midgut under different physiological status.

	Mean concentration ± SE (µg/ml)
Bacteria-infected meal-fed midgut	$114.57 \pm 9.97^{a*}$
Sugar fed midgut	109.43 ± 7.92^{a}

*By ANOVA, numbers with the same letter do not show a significant difference, $P < 0.05$.

L. brevis and *L. reuteri* revealed considerable differences in band patterns. At least four bands were specifically found in the first (BSP1, BSP2, BSP3 and BSP4) and in the second (RSP1, RSP2, RSP3 and RSP4). Additionally, at least twenty bands identified in *L. brevis* were found in *L. reuteri*. Seven of these shared bands (4, 5, 8, 9, 10, 11 and 12) were darker in the profile of *L. brevis*, whereas other seven (1, 2, 3, 7, 13, 14 and 15) were darker in that of *L. reuteri*. Five other shared bands (SM1, SM2, SM3, SM4 and SM5) appeared similarly in both profiles (Figure 1). Overall, the two bacteria do not have the same protein profiles.

Bacterial infections and the synthesis of high molecular weight midgut proteins

The comparison of the synthesis profile of highly weighing proteins between non-infected and *L. brevis*-infected midguts almost showed a similar pattern. No specific band was identified in both profiles. At least fifteen bands were shared between the two profiles. Except one protein (Figure 2, band 12), which was lighter following the uptake of the infected meal, all shared bands had a similar appearance. This suggests that the infection by *L. brevis* has a low effect on the midgut protein synthesis (Figure 2).

The comparison of the synthesis profile of high weight proteins between non-infected and *L. reuteri*-infected midguts revealed considerable differences. The profile from infected females had a greater number and darker bands. At least thirteen bands were shared between the two profiles. Apart from one protein (Figure 3, band 8), females produced greater amount of all shared proteins following the ingestion of the infected meal. This suggests that the infection by *L. reuteri* induced an increase in midgut protein synthesis (Figure 3).

Bacterial infections and the synthesis of low molecular weight midgut proteins

The comparison of the synthesis profile of low molecular weight proteins between non-infected, *L. brevis*-infected and *L. reuteri*-infected midguts showed clear differences in pattern of synthesis. Height bands identified in *L. brevis* were found in *L. reuteri*. The profile of *L. reuteri*-infected midguts showed slightly darker bands than that of *L. brevis*-infected midguts, which, in turn, showed lighter bands than that of non-infected midguts. Female midguts infected with *L. reuteri* synthesized greater amount of they shared with non-infected midguts. In contrast, females midguts infected with *L. brevis* produced lower amount of these shared proteins. Two proteins (Figure 4, bands 5 and 8) seem absent from *L. brevis*-infected midgut. Clearly, the two infections differently affect the synthesis of low molecular weight midgut proteins: *L. reuteri* tended to activate whereas *L. brevis* tended to repress the process (Figure 4).

DISCUSSION

The rearing procedure resulted in similar midgut protein content between infected and healthy *A. albopictus* females. This is likely to suggest that the females used in this study were reasonably similar in fitness. Experimental mosquitoes were treated with erythromycin, an antibiotic which has been shown to have bactericidal activity over many other antibiotics (Vázquez-Martínez et al., 2004). In comparison to the non-treated group, the midguts of antibiotic-treated mosquitoes showed a low prevalence of *Lactobacillus sp*. While the overall effects of antibiotic treatment were considerable, it should be noted that some treated specimens were infected, thus suggesting contamination. This is very important for

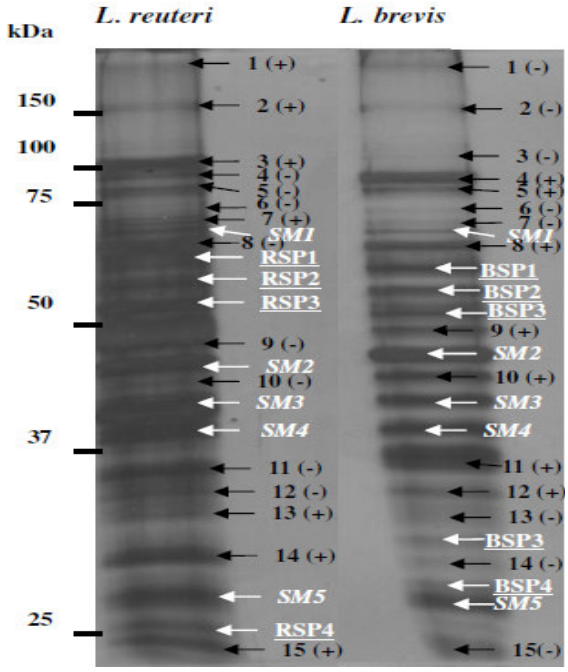

Figure 1. Comparison of protein synthesis patterns between *L. reuteri* and *L. brevis*. M, Precision Plus Proteins™ markers: ~40.0 × 10^6 cfu per ml in 250 µl of lysis buffer, and aliquots of 1.5 µg of proteins were loaded in each lane; SM, similar expression level; (+), high level of synthesis; (-), low level of synthesis; RSP, proteins specific to *L. reuteri*; BSP, proteins specific to *L. brevis*. A 13% polyacrylamide gel was used.

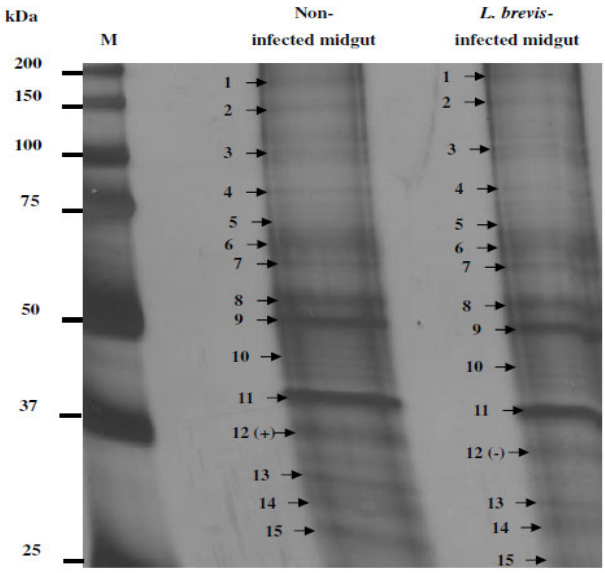

Figure 2. Comparison of protein synthesis patterns between non-infected and *L. brevis*-infected midguts of female *A. albopictus* mosquitoes. M, Precision Plus Proteins™ markers: 24 midguts in 80 µl of lysis buffer, and aliquots of 1.5 µg of proteins were loaded in each lane; (+), high level of synthesis; (-), low level of synthesis. A 13% polyacrylamide gel was used.

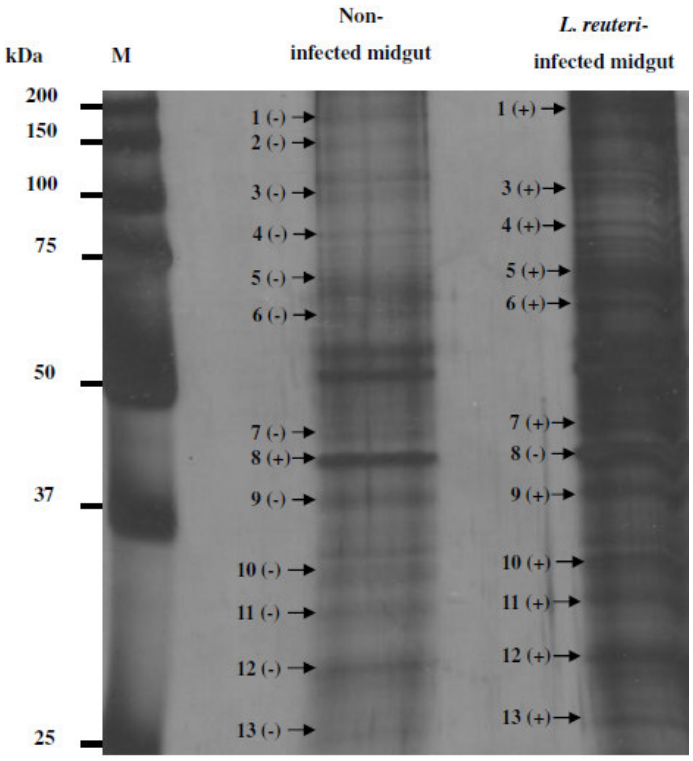

Figure 3. Comparison of synthesis patterns of high molecular weight proteins between midguts of non-infected and *L. reuteri*-infected female *A. albopictus* mosquitoes. M, Precision Plus Proteins™ markers: 24 midguts in 80 µl of lysis buffer, and aliquots of 1.5 µg of proteins were loaded in each lane; (+), high level of synthesis; (-), low level of of synthesis. A 13% polyacrylamide gel was used.

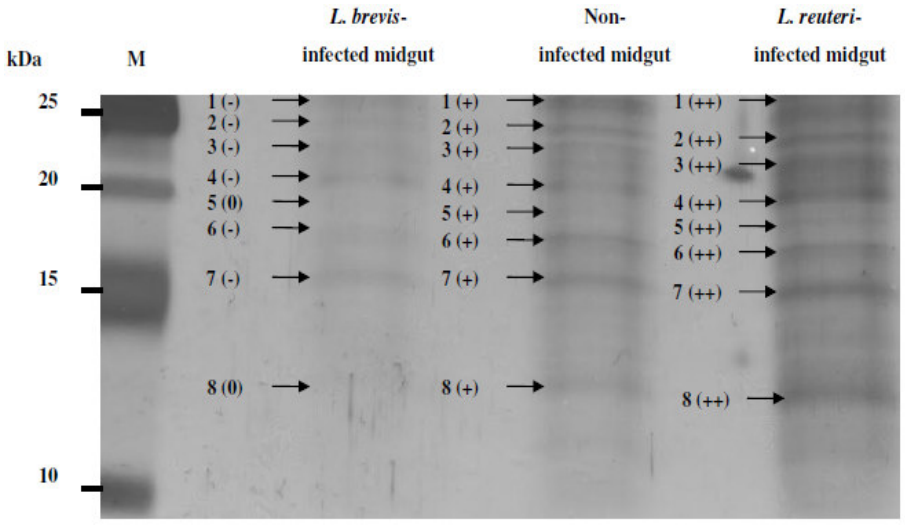

Figure 4. Comparison of synthesis patterns of low molecular weight proteins between midguts of non-infected, *L. brevis*-infected, and *L. reuteri*-infected female *A. albopictus* mosquitoes. M, Precision Plus Proteins™ markers: 24 midguts in 80 µl of lysis buffer, and aliquots of 1.5 µg of proteins were loaded in each lane; (++) indicates a very high level of synthesis; (+), high level of synthesis; (-), low level of synthesis and (0), very low level of synthesis or absence of protein. A 16% polyacrylamide gel was used.

bio-control efforts and is a major requirement if one wants to use a bacterium in a paratransgenic approach to control insect pests that do not have known symbiotic *Lactobacillus* associations. This is typical to number of *Aedes* species, in particular *A. albopictus* known to breed in any discarded non-biodegradable plastics containers with rainwater (Dieng et al., 2002) and not naturally harboring *Lactobacillus*. With a high infectivity, the number of infected individuals will increase, thus aiding to a high colonization under field conditions. Owing to probiotic effects in many animals, lactobacilli are extensively used in biotechnological operations (Hillesland et al., 2008) and commercialized as plastic containers. Methanethiol and isovaleric acid they produce, are very strong attractants to mosquitoes (Braks et al., 1999; Meijerink and van Loon, 1999). This may be useful in encouraging colonization of *Aedes* mosquitoes in a paratransgenic application using *Lactobacillus sp*.

However, ease of infection may not be the single potential strategy by which control efficiency can be improved. An ideal bacterium for paratransgenics would elicit a strong immune response expected to suppress other bacteria and pathogens without affecting its own survival (Lindh et al., 2006). The midguts of mosquitoes infected with *L. brevis* showed lower levels of protein synthesis than those infected with *L. reuteri*. Infection by *L. brevis* or *L. reuteri* tended to down- or up-regulate midgut protein synthesis relative to healthy controls, respectively. In fact, insects are known to mount potent innate immune responses to infection by bacteria. In Drosophila, *Pseudomonas* infection is reported to alter gene expression (Vodovar et al., 2005) and to suppress defense responses (Apidianakis et al., 2005). In lepidopterans, infection by *P. luminescens* resulted in the transcriptional activation of several immune-regulated genes (Eleftherianos et al., 2006). In mosquitoes, Gao and Fallon (2008) reported that cells challenged with bacteria secreted an induced protein that cross-reacts with anti-chicken egg lysozyme antibody. Although no immunological tests were performed, most proteins of molecular weight comprised between 25 and 10 kDa identified in the non-infected midguts were highly produced upon *L. reuteri* infection in contrast to *L. brevis* infection. This range uncovers the molecular weight of most immunogenic proteins and one protein (Figure 4, band 8++) highly present in the midgut following *L. reuteri*-infection, had a molecular weight similar to that of defensin. Lactobacilli are normal components of the human microbiota (Wood and Holzapfel, 1995) and their biochemical processes include proteolysis and production of antimicrobial agents (Daly et al., 1996; Salminen et al., 1996). Presumably, these processes are more pronounced in *L. reuteri*. Thoelen et al. (2008) reported that pathogenicity is closely related to the proteome in *Erwinia amylovora*, based on the results in a study using two moderately pathogenic strains, one highly pathogenic strain, and one very highly pathogenic strain. Comparison of the profiles indicated that one set of proteins were expressed at higher levels in the moderately pathogenic strains, while others showed stronger expression in the highly virulent strain. Probably the proteins specifically present in *L. reuteri* proteome (Figure 1, bands RSP1, RSP2, RSP3 and RSP4) and those shared with *L. brevis* which were highly produced (Figure 1, bands 1, 2, 3, 7, 13, 14 and 15) are involved in defensive processes.

Typically, a paratransgenic approach would utilize bacteria capable of persisting in the mosquito midgut. This prerequisite is in line with the observation showing the presence of *L. casei* populations in the midgut of *A. albopictus* 5 days after the infected meal was taken. Although we did not assess the size of these populations, the important point is that by the time midgut dissections were performed, a virus in the ingested meal would have interacted with bacteria, which means that they can resist altering lumen conditions, such as pH, digestive enzymes and the immune system.

Both *L. brevis* and *L. reuteri* improve human immune function, but the latter likely has greater immune-potentiating activity on the host cell-mediated and humoral immune responses (Casas et al., 1998). Based on these immunological potentials and given the low levels of expression due to *L. brevis*, it seems likely that *L. reuteri* is suitable as a paratransgenic tool. Although *L. reuteri* infection resulted in increased levels of protein synthesis in the midgut it is important that it is not pathogenic to the recipient. As part of the paratransgenic approach, it will be necessary to deliver transformed bacteria to the recipient to allow for colonization and persistence. There is a close association of humans and mosquitoes such as dengue vectors, necessitating the use of human non-pathogenic bacteria, as it is being proposed here.

Slight difference in band pattern is quite common during electrophoresis without any specific reason because of the sample preparation etc. Here, although protein analysis was conducted with one-dimensional electrophoresis that used a fixed protein amount, profiles were clearly different between bacteria species and between infected (*L. reuteri* and *L. brevis* infections) and non-infected midguts. This suggests that our technique was adequate and that the obtained protein expression patterns may not be the result of artifacts. These commonly found bacteria are highly likely ideal candidates that could be used toward paratransgenic control of dengue. This is mandatory for their genetic engineering to express anti-dengue virus compounds.

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