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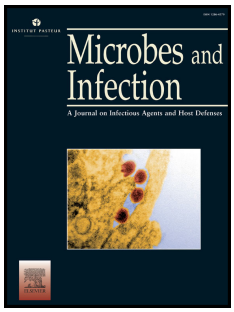

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Cross-reactivity and immunotherapeutic potential of BamA recombinant protein from *Acinetobacter baumannii*

Anna Erika Vieira de Araujo[a,b], Luis Vidal Conde[a], Haroldo Cid da Silva Junior[a], Lucas de Almeida Machado[b], Flávio Alves Lara[b], Alex Chapeaurouge[b], Heidi Pauer[c], Cristiane C. P. Hardoim[d], Luis Caetano M. Antunes[b,c], Ana Paula D'Alincourt Carvalho-Assef[b], Jose Procopio Moreno Senna[a]*

[a]Bio-Manguinhos, Oswaldo Cruz Foundation, Brazilian Ministry of Health, Rio de Janeiro, RJ, Brazil. [b]Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Brazilian Ministry of Health, Rio de Janeiro, RJ, Brazil. [c]National Institute of Science and Technology of Innovation on Diseases of Neglected Populations, Center for Technological Development in Health, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil. [d]São Paulo State University, Institute of Biosciences, Coastal Campus of São Vicente, São Paulo, SP, Brazil.

Email Adresses:

Anna Erika Vieira de Araujo: anna.vieira@bio.fiocruz.br

Luis Vidal Conde: lvcbcn40@gmail.com

Haroldo Cid da Silva Junior: haroldo.cid@bio.fiocruz.br

Lucas de Almeida Machado: biolucasmachado@gmail.com

Flavio Alves Lara: falara@ioc.fiocruz.br

Alex Chapeaurouge: donat.chapeaeurouge@fiocruz.br

Heidi Pauer: heidipauer@gmail.com

Cristiane Cassiolato Pires Hardoim: cristianehardoim@gmail.com

Luis Caetano Martha Antunes: caetano.antunes@fiocruz.br

Ana Paula D'Alincourt Carvalho-Assef: apdca@ioc.fiocruz.br

26 Jose Procopio Moreno Senna: jprocopio@bio.fiocruz.br

27

28 **Corresponding author:**

29 Jose Procopio Moreno Senna (jprocopio@bio.fiocruz.br)

30 Avenida Brasil 4365, Manguinhos, Rio de Janeiro-RJ. 21.040-900.

31 Telephone number: +552138829516

Abstract:

Acinetobacter baumannii is an important nosocomial pathogen. BamA is a protein that belongs to a complex responsible for organizing the proteins on the bacterial outer membrane. In this work, we aimed to evaluate murine immune responses to BamA recombinant protein (rAbBamA) from *A. baumannii* in an animal model of infection, and to assess cross-reactivity of this target for the development of anti-*A. baumannii* vaccines or diagnostics. Immunization of mice with rAbBamA elicited high antibody titers and antibody recognition of native *A. baumannii* BamA. Immunofluorescence also detected binding to the bacterial surface. After challenge, immunized mice demonstrated a 40% survival increase and better bacterial clearance in kidneys. Immunoblot of anti-rAbBamA against other medically relevant bacteria showed binding to proteins of approximately 35 kDa in *Klebsiella pneumoniae* and *Escherichia coli* lysates, primarily identified as OmpA and OmpC, respectively. Altogether, our data show that anti-rAbBamA antibodies provide a protective response against *A. baumannii* infection in mice. However, the response elicited by immunization with rAbBamA is not completely specific to *A. baumannii*. Although a broad-spectrum vaccine that protects against various pathogens is an appealing strategy, antibody reactivity against the human microbiota is undesired. In fact, immunization with rAbBamA produced noticeable effects on the gut microbiota. However, the changes elicited were small and non-specific, given that no significant changes in the abundance of Proteobacteria were observed. Overall, rAbBamA is a promising target, but specificity must be considered in the development of immunological tools against *A. baumannii*.

Keywords: *Acinetobacter baumannii*; BamA; immunogenic; specificity; resistant bacteria

1. Introduction

Acinetobacter baumannii is a Gram-negative coccobacillus and important opportunistic pathogen responsible for several outbreaks of nosocomial infections in hospitals around the world [1]. Its rapid emergence and global spread demonstrates the successful adaptation of this pathogen in the hospital environment [2]. Treatment of multiresistant *A. baumannii* strains is generally limited to carbapenems, but the emergency of imipenem and meropenem resistant strains is a major concern [3].

Therefore, with the disturbing gradual increased incidence of infections caused by strains with this antimicrobial resistance pattern, it is necessary to expand the therapeutic options to prevent and treat infections caused by this pathogen. In this context, immunotherapies are considered as great alternatives to typical antibiotic treatments [4].

The search for new targets for the development of immunotherapeutic strategies usually focuses on virulence and colonization factors located on the bacterial surface, such as outer membrane proteins (OMPs) [5]. These proteins are important *in vivo* virulence factors for *A. baumannii*, specially the major component OmpA, which can induce apoptosis upon binding to epithelial cells and is involved in biofilm formation [6]. In previous reports, active immunization with OmpA, OmpW and Omp22 elicited immune and protective responses to *A. baumannii* [7–9]. Likewise, OmpA was previously investigated as a considerable target for other Gram-negative bacteria, such as *E. coli*, *Haemophilus parasuis* or *Leptospira interrogans* [10–12].

Interestingly, the formation and organization of OMPs in the bacterial membrane occurs mainly through the action of the Bam protein complex (β-Barrel Assembly Machinery), which is essential for bacterial survival [13]. Among these proteins, BamA represents a potential target, primarily due to the fact that it is anchored to the cell membrane, with a small extracellular portion that can generate immunogenic epitopes [14]. In addition, *in*

silico and experimental analyses demonstrated that *A. baumannii* BamA is a good vaccine candidate [15,16]. Other studies in *E. coli* also suggested the bactericidal potential of anti-BamA antibodies [17]. Thus, we aimed to analyze the immunogenicity of recombinant *A. baumannii* BamA in a murine model and to evaluate the specificity of the immune response elicited by testing cross-reactivity with other Gram-negative bacilli and assessing the impact on the resident microbiota. The results presented herein validate BamA as a promising target for immunotherapies or immunodiagnostics against infections caused by *A. baumannii*.

2. Material and Methods

2.1. Cloning, expression and purification of recombinant BamA protein from Acinetobacter baumannii (rAbBamA)

PCR amplification of the *bamA* gene was performed (forward primer: ATTGCTAGCATGCGGCACACACATTTTTTAATGCCTTTG; reverse primer: TACGTCGACTTAGAAAGTACGACCAATTT) to generate a 2526-base pair (bp) amplicon from *A. baumannii* ATCC19606 genomic DNA. Primers were designed based on the sequence available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (GeneID: 31351149). The sequence was cloned into pET28a vector (Novagen, USA) with NheI/SalI restriction enzymes. Subsequently, BL-21(DE3) *Escherichia coli* cells (Life Technologies, USA) were transformed with the recombinant plasmid and induced with 50 µM IPTG at 37°C for 4 h. The cells were harvested, resuspended in denaturation buffer (6 M urea, 20 mM Tris, 500 mM NaCl, 5 mM imidazol; pH 8.0), and disrupted by sonication (3 × 1 min pulses; Sonics & Material Inc). After sample clarification, the soluble fraction was subjected to immobilized metal ion affinity chromatography (IMAC), using a His Trap HP column (GE Healthcare, UK). rAbBamA was eluted from the column with a linear gradient of denaturation buffer containing 500 mM imidazole. To promote refolding, purified rAbBamA

was rapidly diluted in phosphate buffered saline (PBS)-Tween 20 (0.1%) to a concentration of 0.5 mg/mL and placed at 37°C for 18 h [18]. Afterwards, in order to remove urea and imidazole salts, the recombinant protein was then dialyzed in PBS-Tween 20 0.1%. Collected samples were analyzed using SDS-PAGE and quantified *via* spectrophotometric absorbance analysis at 280 nm using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

2.2. Animal studies and ethical statement

Eight-week-old female C57/BL6 mice provided by the *Instituto de Ciência e Tecnologia em Biomodelos* (Fiocruz, Rio de Janeiro, Brazil) were kept at the Animal Experimentation Laboratory (LAEAN, Bio-Manguinhos) animal house in clean ventilated polysulfone cages (ALESCO, Brazil) with free access to potable water and food (NUVINLAB, Brazil). All animal procedures were approved by the Ethics Committee on Animal Research (CEUA), FIOCRUZ (License number LW-21/18) and were performed in accordance to the guidelines of the National Council for Animal Experimentation Control (CONCEA, Ministry of Science and Technology, Brazil). In animals presenting severe clinical symptoms, a humanitarian endpoint was considered based on suffering or inability to access water or feed, as also recommended by the ARRIVE guidelines.

2.3. Mice immunization protocol

The mice received three intramuscular doses of 25 µg of rAbBamA in 250 µg of aluminum hydroxide (Alhydrogel®, Brentag) at intervals of two weeks. Blood was collected from the retro-orbital plexus prior to the immunizations (preimmune sera) and two weeks after the second and third doses (booster 1 and booster 2 immune sera), respectively. Feces were also collected one day before the immunization doses (preimmune, immune – after booster 1 and 2).

130

131 *2.4. ELISA*

132 In order to evaluate if the immunizations were able to generate antibodies against rAbBamA,
133 an indirect immunoassay was performed as described in Silva-Junior et al. 2017 [19]. Briefly,
134 following secondary antibody incubation (1:30,000 (v/v) in PBS 1% non-fat milk of anti-
135 mouse IgG HRP conjugated, Sigma-Aldrich), reaction development was performed with the
136 addition of a chromogenic substrate solution (TMB peroxidase, Bio-Rad Laboratories Inc.).
137 After the reaction was stopped (by addition of 2 N H_2SO_4; 50 µL/well), the optical density
138 (OD) was read on a microplate reader (Sunrise™, Tecan) at 450 nm. Cut-off values were
139 determined as three times the average of preimmune sera.

140

141 *2.5. Western Blot*

142 rAbBamA or *A. baumannii* bacterial culture lysates were subjected to electrophoresis in 12%
143 denaturing polyacrylamide gel (SDS-PAGE) and transferred to a 0.2 µm nitrocellulose
144 membrane [20]. Proteins were detected using the color reagent Western Blue® Stabilized
145 Substrate for Alkaline Phosphatase (Promega), until bands were visualized. The reaction was
146 stopped upon the addition of distilled water.

147

148 *2.6. Fluorescence microscopy*

149 A bacterial inoculum (5×10^7 CFU/mL) of a clinical *A. baumannii* strain (AB162) was grown
150 in Luria-Bertani medium (37°C, 200 RPM) until exponential phase (OD_{600} 0.4). Preimmune
151 mice sera was used as negative control. Non-specific targets were blocked by incubation with
152 a solution of PBS BSA 0.1% and human IgG 5 µg/mL (Sigma-Aldrich) for 30 minutes at 4°C.
153 Washing was performed between incubation steps with PBS BSA 0.1%. Crude anti-
154 rAbBamA (50 µL) mice serum was added as primary antibody for two hours at 4°C. Diluted

rabbit IgG anti-mouse–Alexa Fluor 633 (Thermo Fisher Scientific Inc.) (1:200 in blocking solution) conjugates were added as secondary antibodies for an additional two hours at 4ºC. Samples were immediately analyzed using a conventional light microscope (Zeiss Axio Observer Z1, Carl Zeiss, Heidenheim, Germany) with a Zeiss Plan-Apochromat 100x objective. Excitation was achieved using an HXP-120 light source with a Zeiss Filter Set 50. Images were acquired using a Zeiss HMRc CCD camera controlled by AxioVision version 4.8 software.

2.7. In vivo mice studies

2.7.1. Bacterial strains and growth conditions

Culture conditions were as previously described [21]. In order to reduce the influence of LPS-toxicity to mice, a mucin model was used. Briefly, overnight cultures of *A. baumannii* clinical strain AB162 [22] were diluted 1:100 with fresh LB medium, and grown at 37°C 200 rpm until OD_{600} of 1.0. Cultures were diluted to the appropriate bacterial inoculum concentration (between 5 x 10^3 and 1 x 10^4 CFU) in sterile PBS containing 2.5% mucin (Sigma-Aldrich M1778). Bacterial concentrations in the inoculum were determined by plating 10-fold dilutions on LB agar. The inoculum was administered intraperitoneally in immunized and non-immunized C57/BL6 mice (n=10). After 18 h, the animals were euthanized and their kidneys were removed for assessment of bacterial burden. Alternatively, animals were monitored for seven days to compare the survival rates of immunized *vs* control animals.

2.7.2. Cross-reactivity with other Gram-negative bacteria

Outer membrane proteins (OMPs) were extracted from cultures of *A. baumannii* ATCC19606 and other bacteria of nosocomial importance such as *Pseudomonas aeruginosa* (ATCC17853), *E. coli* (ATCC25922) and *Klebsiella pneumoniae* (ATCC14700) with a previously described method using 4% Triton X-114 and protein precipitation with cold

acetone [5]. The proteins were verified through SDS-PAGE and blotted to a nitrocellulose membrane according to the described Western Blot method.

2.7.3. Mass spectrometry protein identification

Antibody-recognizable protein bands were excised from SDS-PAGE and treated according to protocol [5]. Protein identification was performed on an AB Sciex 5800 (AB Sciex, Foster City, CA) MALDI mass spectrometer; detailed parameters are described in Fajardo-Bonin et al. 2014 [5]. Finally, the tandem mass spectra were searched against all entries of NCBI (http://www.ncbi.nlm.nih.gov/index.html) using the Mascot software (http://www.matrixscience.com). The following search parameters were used: no restrictions on species of origin or protein molecular mass, tryptic cleavage products, two tryptic missed cleavages allowed, variable modifications of cysteine (carbamidomethylation), asparagine and glutamine (deamidation), methionine (oxidation), and pyroglutamate formation at the N-terminal glutamine of peptides.

2.8. DNA extraction from feces

DNA was isolated from approximately 200 mg of feces using the QIAamp DNA Stool Minikit (Qiagen, Hilden, Germany) with modifications. Mechanical lysis was performed by transferring the samples to PowerBead Tubes, Garnet (Qiagen, Hilden, Germany), adding 1 mL of InhibitEX Buffer to each sample and incubating the tubes at 95ºC for 5 minutes. Then the tubes were placed horizontally in a vortex adapter and vortexed at maximum speed for 10 min. The content of the tubes was centrifuged at 13,000 x *g* for 1 min and the supernatant was transferred to a clean 2 mL microtube. The next steps were performed according to the manufacturer's instructions. DNA was stored at -80 ºC until analysis.

2.9. 16S rDNA sequencing

We used standard Illumina protocols for the preparation and sequencing of 16S rDNA amplicon libraries. For this, variable regions V3 and V4 of the 16S rRNA gene were amplified using primers 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
(forward) and
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
(reverse) and KAPA HiFi HotStart ReadyMix enzyme (Roche, Pleasanton, USA). This reaction creates amplicons containing protruding adapter strings for compatibility with the Illumina index and sequencing adapters. After preparation, libraries were sequenced on the MiSeq system with chemistry v2 500 cycles.

2.10. Analyses of sequencing data

Mothur v. 1.44 was used to process the Illumina sequences [23]. A pipeline was optimized and executed. Briefly, paired raw reads were subjected to quality checking and then reduced to unique sequences. The reference SILVA seed v. 138 database (mothur-formatted), provided by Mothur was used to align the sequences [24,25] . Once again, the data set was reduced to non-redundant sequences and pre-clustered. UCHIME was applied to identify chimeric sequences, which were removed from the data set [26]. Sequences were then phylogenetically classified. Based on the classification, undesirable sequences (Mitochondria-Chloroplast-Eukaryota-unknown) were removed from the data set. Then, sequences were assigned to operational taxonomic units (OTUs) at 97% sequence similarity. Afterwards, singletons were removed and the OTU table was sub-sampled to normalize libraries to 11,274 reads per sample. OTUs were further classified based on the SILVA non-redundant v. 138 database (mothur-formated). All 16S rRNA data sets generated in this study were deposited as

Sequence Read Archive in NCBI database with Bioproject ID: PRJNAXXXX (SAMNXXXXXX-SAMNXXXXXX). A description of the scripts used in this pipeline can be found in the Supplementary Material (S1). The OTU table generated was then processed using the MicrobiomeAnalyst online software suite (https://www.microbiomeanalyst.ca/) in Marker Data Profiling mode.

2.11. Statistical analysis

Statistics were performed using GraphPad Prism software, version 5. Non-parametric Mann-Whitney U test was used for bacterial burden analysis. Survival results were plotted as Kaplan–Meier curves and analyzed with log-rank tests. Alpha diversity data was analyzed using the Kruskal-Wallis test with multiple comparison FDR correction with the Benjamini, Krieger and Yekutiely test. Beta diversity was analyzed using the Bray-Curtis index as the distance method and PERMANOVA. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Synthesis of recombinant protein (rAbBamA)

After PCR amplification of the *A. baumannii* genomic DNA it was possible to observe a single band at approximately 2500 bp, which corresponds to the size presented by the *bamA* gene (2526 bp) (Fig. 1a). Subsequent transformation to *E. coli* BL21 (DE3) and IPTG induction for 4 hours led to the abundant expression of an approximately 96-kDa protein, which relates to the expected molecular weight of rAbBamA (Fig. 1b). As expected, since this is a transmembrane protein, rAbBamA presented low solubility, being expressed mainly in the form of inclusion bodies (Fig. 1b). Therefore, due to this characteristic, we added a denaturing agent before the purification step by metal ion affinity chromatography (IMAC) in order to solubilize inclusion bodies. After chromatography, a homogenous fraction containing

a major band of 96 kDa was obtained, corresponding to rAbBamA (Fig. 1c). After refolding, protein samples were adjusted to the concentration of 0.5 mg/mL and stored at -20°C.

Fig. 1

3.2. Immunogenicity of rAbBamA in mice

After mice immunizations with rAbBamA, indirect ELISA showed that the immunization generated high antibody titers (up to 1:256,000), especially when compared to preimmune serum absorbance values (Fig. 2a).

In order to confirm the results observed in the ELISA assays and to verify the specificity of the sera generated by immunization with rAbBamA, immunoblotting assays were performed. Here, immunizations induced antibodies against rAbBamA and these antibodies were able to recognize a single protein band with a similar molecular weight (approximately 96 kDa) in the bacterial lysate of *A. baumannii* (AB162 strain) (Fig. 2b). No recognizable protein band was noticed when preimmune sera were used. These results suggest that the anti-rAbBamA antibodies can recognize both the recombinant and native proteins.

Fig. 2

However, since the electrophoresis carried out to transfer proteins from lysates to the nitrocellulose membrane was performed under denaturing conditions, recognition of these anti-rAbBamA antibodies directly on the bacterial surface must be evaluated. For that reason, an immunofluorescence assay (IF) was performed.

In the IF assays, non-p-formaldehyde treated bacteria were used in order to maintain the internal structures as preserved as possible and to observe the direct interaction of antibodies with the bacterial surface. *A. baumannii* AB162 cultures were grown to exponential phase and then incubated with anti-rAbBamA mice serum. Non-immunized animal serum served as

negative control. Captured images demonstrated that immunizations with rAbBamA induced the production of antibodies that recognized proteins on the bacterial surface (Fig. 3).

Fig. 3

3.3. In vivo protection studies

Two weeks after the third immunization dose (booster 2), groups of ten C57BL/6 mice received an intraperitoneal inoculum of $6x10^3$ CFU of a clinical *A. baumannii* strain (AB162) in 2.5% mucin. Since these strains are notably more virulent, their use is preferable to an ATCC. This inoculum was sub-lethal since almost all the animals survived the challenge, except one animal from the control group (non-immunized), who died after 18 hours (n = 10). Previous tests showed that there was not much discrepancy between quantification of bacteria in different organs, such as kidneys and spleen, therefore only renal quantification was evaluated (data not shown). Here, it was possible to observe a bacterial load reduction in the majority of mice, with some varying four logs (Fig. 4a). Although there is a variability between two animals of the non-immunized group, the decrease tendency is notorious.

Concerning the animal survival rate between immunized and non-immunized groups (n = 10), an inoculum of $1x10^4$ CFU in 2.5% mucin was given intraperitoneally. After 7 days, there was a 40% increase in animal survival of rAbBamA-immunized animals when compared to the non-immunized group, indicating a rather protective role of this protein as a vaccine (Fig. 4b). Interestingly, all deaths occurred in the first 24 hours after bacterial inoculation, demonstrating the fast induction of sepsis in this animal model.

Fig. 4

3.4. Cross-reactivity of anti-rAbBamA with other Gram-negative bacteria

Sequence analysis of *A. baumannii* BamA demonstrates the protein is rather conserved in the *Acinetobacter* genus. However, since BamA is a membrane protein that presents homology

306 with similar functional proteins of other Gram-negative bacteria, it is important to evaluate
307 the specificity of this protein as an immunogenic target. Here, cross-reactivity of anti-
308 rAbBamA antibodies was assessed against OMPs of other bacteria with great importance in
309 hospital-acquired infections, such as *E. coli, K. pneumoniae* and *P. aeruginosa*. ATCC strains
310 were chosen because they are more thoroughly characterized and can be tracked to the source.
311 After extraction and solubilization, OMPs from these bacteria were subjected to SDS-PAGE
312 and Western blot with anti-rAbBamA polyclonal sera. These antibodies showed reactivity
313 with proteins at 35 kDa regions for *E. coli* and *K. pneumoniae* (Fig. 5).

314 Fig. 5

315 Nevertheless, no binding was noted for *P. aeruginosa* proteins or against preimmune sera.
316 MALDI-TOF/MS analysis resulted in peptide identification of these bands as the major
317 OMPs proteins OmpA and OmpC for both *E. coli* and *K. pneumoniae*. These proteins do not
318 contain a high identity degree with rAbBamA, but they are highly abundant and
319 immunogenic, presenting matching peptides sequences that could function as epitopes (Table
320 S1).

321

322 *3.5.The effect of immunization with rAbBamA on the murine gut microbiota*

323 Due to the detectable cross-reactivity of anti-rAbBamA polyclonal sera against related
324 proteins of other bacterial species described above, we sought to determine if immunization
325 with rAbBamA resulted in drastic changes to the composition of the murine gut microbiota.
326 Given the role of the gut microbiota in human health [27], cross-reactivity-induced shifts in
327 microbiota composition could be a detrimental effect of immunization and should be taken
328 into account when designing immunotherapeutics against BamA. To determine gut
329 microbiota compositions of preimmune and immunized animals (including animals who
330 received one or two booster doses), we collected fresh feces from these animals, extracted

fecal DNA, amplified the V3-V4 region of the 16S rRNA gene, and sequenced the amplicons using an Illumina MiSeq system. By doing so we obtained a combined total of 169,110 sequences after Mothur analysis, with an average of 11,274 sequences per animal (9,780-10,748 range). As can be seen in Fig. 6a, Principal Coordinate Analysis (PCoA) showed that the microbiota of preimmune animals is significantly distinct from those of immunized animals ($p<0.001$). Alpha diversity values were determined for each animal and, interestingly, diversity was significantly increased in immunized animals, as can be seen in Fig. 6b.

Fig. 6

However, although detectable changes in microbiota composition after immunization with rAbBamA could be observed, these changes were relatively minor, and Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis using FDR-corrected $p<0.1$ as cut-off failed to produce OTUs whose relative levels were significantly altered by immunization. However, by loosening the stringency of the analysis (non-FDR-corrected $p<0.05$ as cut-off), we detected 12 OTUs (genus-level) that could be significantly associated (LDA score >4, $p<0.05$) with one of the sample groups (preimmune, booster 1 and booster 2) (Fig. 6c). Interestingly, though, none of the OTUs found were assigned to the phylum Proteobacteria, the most likely bacterial group to suffer from cross-reactivity of an antibody against an *A. baumannii* protein. In fact, we compared the relative abundance of all OTUs matched to Proteobacteria and did not find significantly different levels when the different animal groups were compared (Fig. 6d). These findings suggest that the effect of immunization on the gut microbiota is mild and is likely not the result of cross-reactivity of anti-rAbBamA against BamA homologues in other Proteobacteria.

4. Discussion

Over the last years, *A. baumannii* has emerged as an important pathogen related to nosocomial infections worldwide. To minimize the impact of this pathogen in the community, there is an urgent need to develop viable alternatives to conventional synthetic antimicrobials, for the treatment or prophylaxis of infections caused by *A. baumannii* [28].

Outer membrane proteins (OMPs) are widely described in the literature as potential immunogenic targets [29]. However, despite the gene deletion of important OMPs significantly affect virulence and fitness, these are not essential proteins for bacterial survival [30,31]. In an opposite manner, architectural proteins, like β-Barrel Assembly Machinery complex proteins, have a primordial effect on securing membrane integrity. Therefore, we proposed to assess the immune and protective responses elicited by the *A. baumannii* BamA protein in a murine mucin-induced sepsis model, as well as anti-sera reactivity to membrane proteins of other medically important Gram-negative bacteria and the subsequent impact on related organisms in the gut microbiota.

In an infection model with a high bacterial inoculum (10^9 CFU), Singh et al. (2017) showed that immunization with recombinant *A. baumannii* BamA was able to induce a protective response. Immunized animals demonstrated an 80% increase in survival and lower bacterial load on kidneys when compared to the non-immunized group [16].

Hence, the results shown here confirm those of the previous study, with a 40% survival increase and reductions of up to four logs of bacterial loads in the kidneys of rAbBamA-immunized animals. Nonetheless, it is important to point out that our results are from a mucin-based mice infection model, with a lower bacterial inoculum (approximately 10^4 CFU). This infection model has the advantages of minimizing LPS influence and better resembling how the infectious process actually occurs in patients colonized by *A. baumannii*, since infection with high bacterial loads (such as 10^8 or 10^9 CFU) are unrealistic [32].

Preferably, an immunogenic target should be able to generate a strong immune response and functional antibodies. Mice immunization with rAbBamA generated high antibody titers, demonstrating the induction of humoral responses normally seen in vaccines that use aluminum salts as adjuvant [33]. Protein antigens are notable inducers of memory T-cells; consequently, a cellular response is also induced in outer membrane protein vaccine preparations and specifically in *A. baumannii* BamA immunizations, with the increase of macrophage opsonophagocytic capacity [16,34].

However, in addition to the results published by Singh et al. (2017), bacterial lysate immunoblotting and fluorescence microscopy assessed the binding of anti-rAbBamA antibodies to the native *A. baumannii* BamA protein. Interestingly, not all bacteria presented antigenic recognition in the immunofluorescence assays. Since AbBamA is a large sized transmembrane protein (approximately 96 kDa), most of the antibody recognition epitopes may be located towards the inner membrane or periplasm of the bacterial cell. Moreover, factors such as the capsule polysaccharide could physically restrain antigenic binding [35]. Limited epitope access can be a problem in an immunotherapeutic approach; however, an anti-LPS vaccine demonstrated subcapsular targeting activity in *K. pneumoniae* strains, a notable capsule forming bacteria [36]. Other studies have also considered this a controversial hypothesis [37,38].

Due to the essential nature of BamA in the architecture of the bacterial outer membrane, the inhibition of this protein by antibodies or immune complexes could lead to killing of bacterial cells [16,17]. Nevertheless, as demonstrated in the *in vivo* assays, the generated anti-rAbBamA antibodies in the polyclonal sera showed to be functionally effective.

Previous studies with an anti-*E. coli* recombinant BamA vaccine detected cross-reactivity for other enteropathogenic bacteria such as *Shigella sp.* and *Salmonella sp.* [39]. Herein, anti-rAbBamA antibodies reacted intensely to *E. coli* and *K. pneumoniae* membrane proteins,

OmpA and OmpC. These bacteria are members from the same family (*Enterobacteriacea*e) and their proteins have a conserved epitope nature [40]. These proteins are acknowledged immunogenic antigens, with several studies demonstrating their potential use in vaccination strategies [10,40–42]. Curiously, anti-rAbBamA polyclonal antibodies did not recognize Opr86, a BamA homolog in *P. aeruginosa* (41% identity with AbBamA), which means that peptide identity does not always reflect in generating effective immune epitopes.

The presence of cross-reactivity may be advantageous for therapeutic purposes [43]. Given the high homology degree between these proteins in Gram-negative bacteria, it would be rather unlikely not to observe this type of response experimentally. A multidirectional strategy may be interesting, although reactivity with microorganisms belonging to the human microbiota must be avoided [44]. To determine if this was indeed the case, we characterized the gut microbiota of animals prior to or after immunization. Although noticeable changes did occur, we did not observe a drastic change in gut microbiota composition between different animal groups. Given the cross-reactivity of anti-rAbBamA polyclonal antibodies with BamA from closely related bacteria, we evaluated the impact of immunization on levels of Proteobacteria in the gut microbiota of these animals. Proteobacteria sequences represented only 0.8% of all sequences obtained, and no significant differences in relative levels of this bacterial group were detected when animals from different treatment groups were compared. Therefore, further evaluation of rAbBamA protein as an immunological target could lead to the development of novel tools against bacterial infections.

In conclusion, the data presented herein demonstrates that immunization with recombinant *A. baumannii* BamA protein provides immunogenic and protective responses in mice. Although these anti-rAbBamA antibodies recognize other bacterial species, they were not relevant enough to cause major shifts in microbiota composition. This protein is still an interesting

antigenic target, and experimental assessment of cross-reactivity to other bacteria must be accomplished, especially if used for immunodiagnostics.

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6. Conflicts of Interest

The authors declare no conflicts of interest.

7. Author Contributions

A.E.V.A performed research and wrote the paper; L.V.C, H.C.S.J, L.A.M, F.A.L., H.P., C.C.P.H. and A.C. performed research; C.C.P.H., L.C.M.A., A.P.D.C.A and J.P.M.S analyzed data and reviewed paper.

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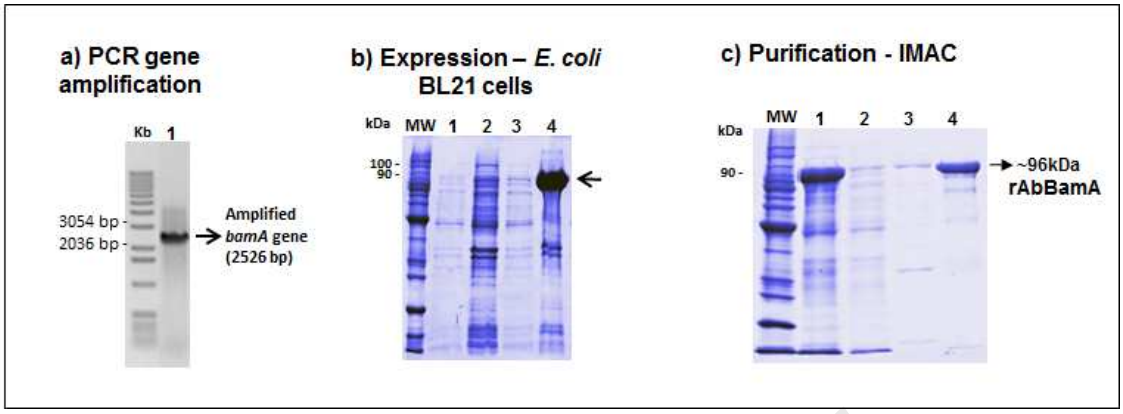
Figure 1:



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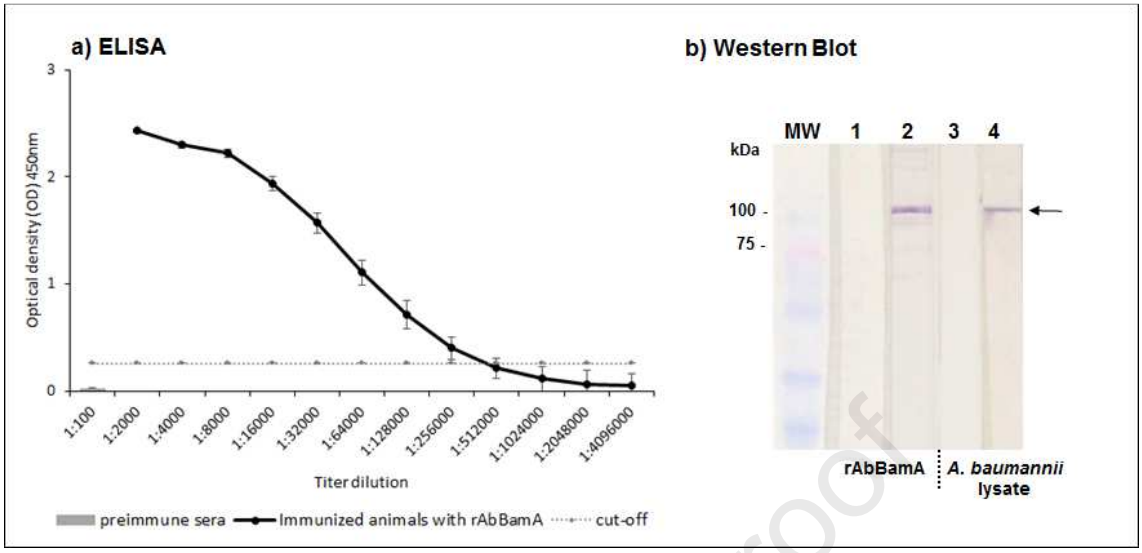

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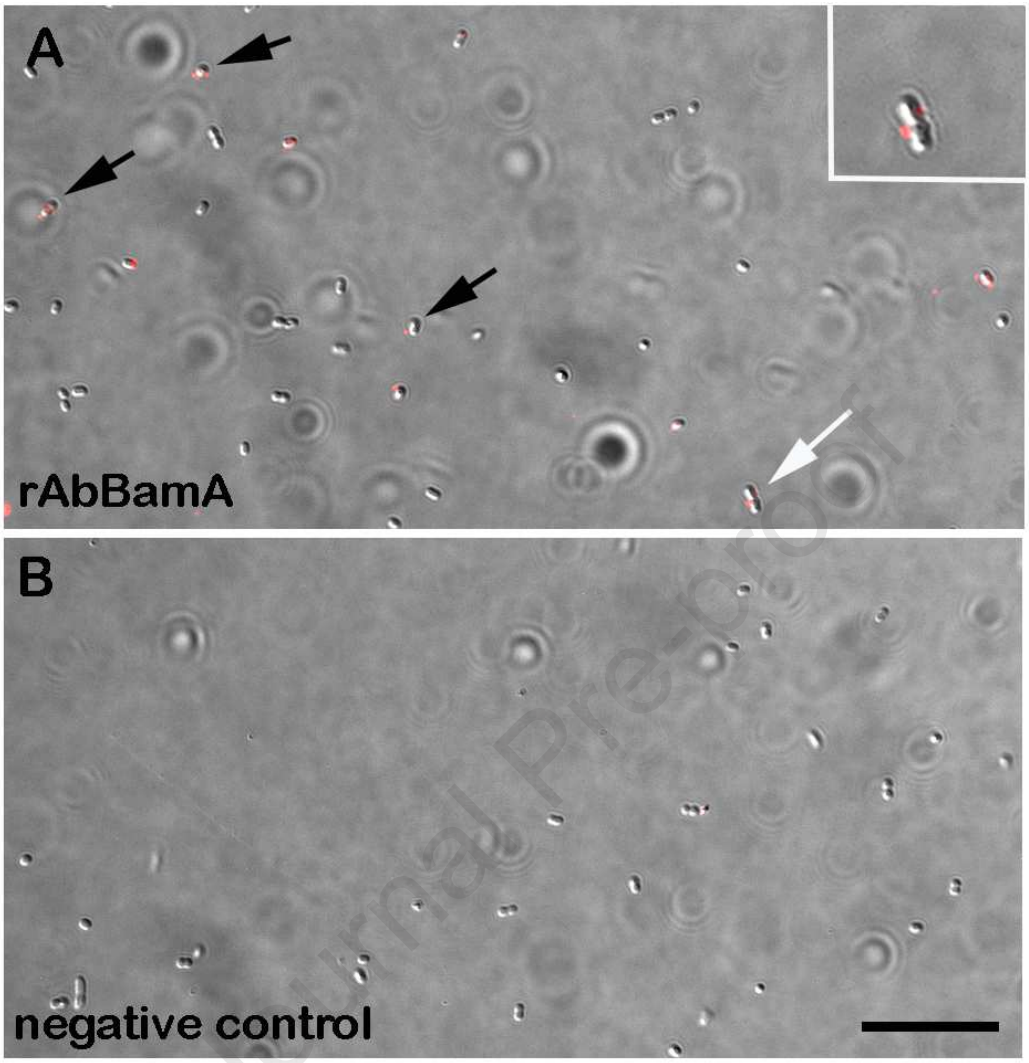

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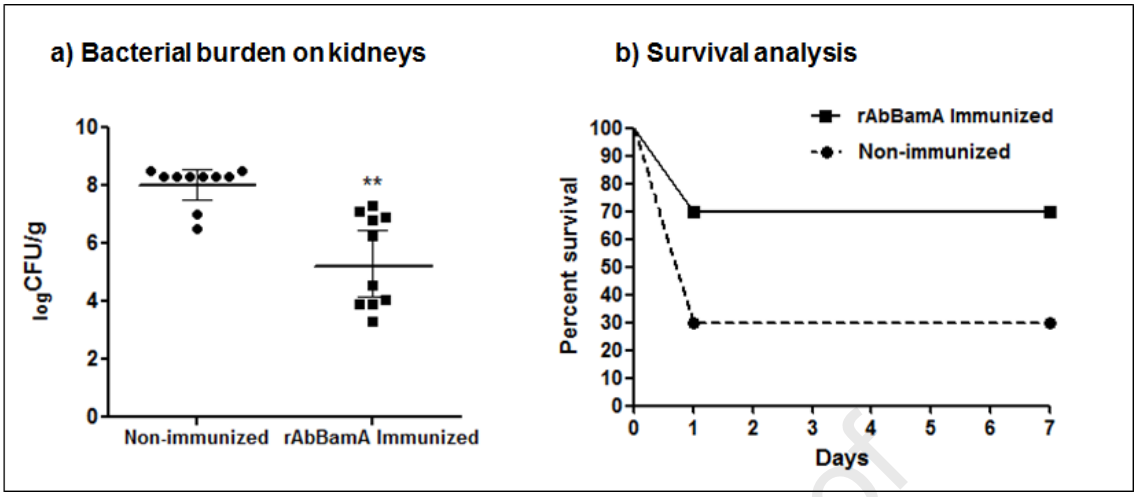

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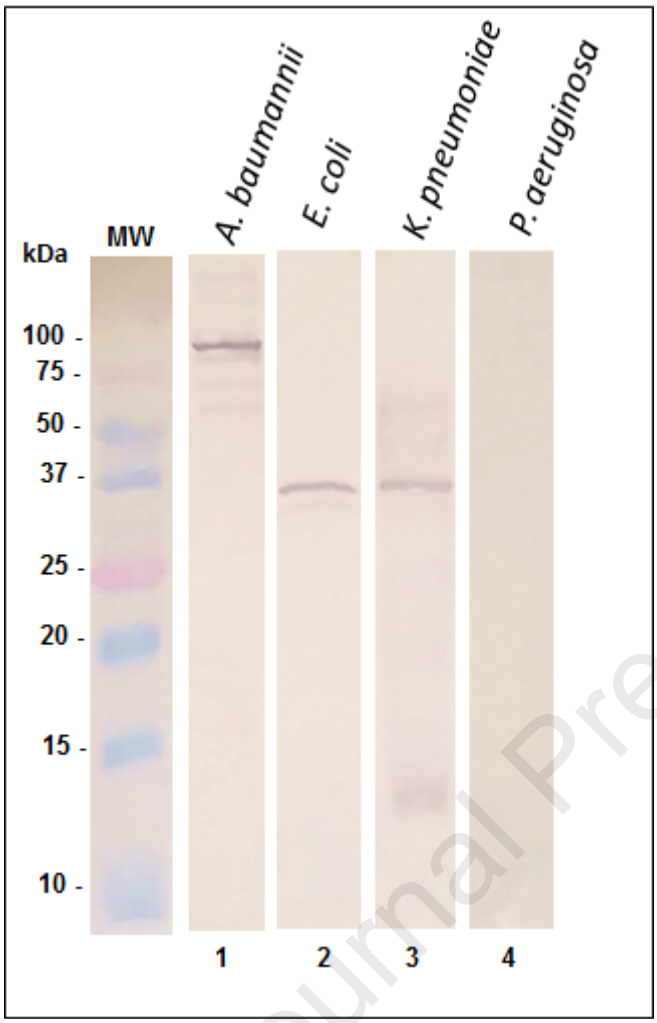

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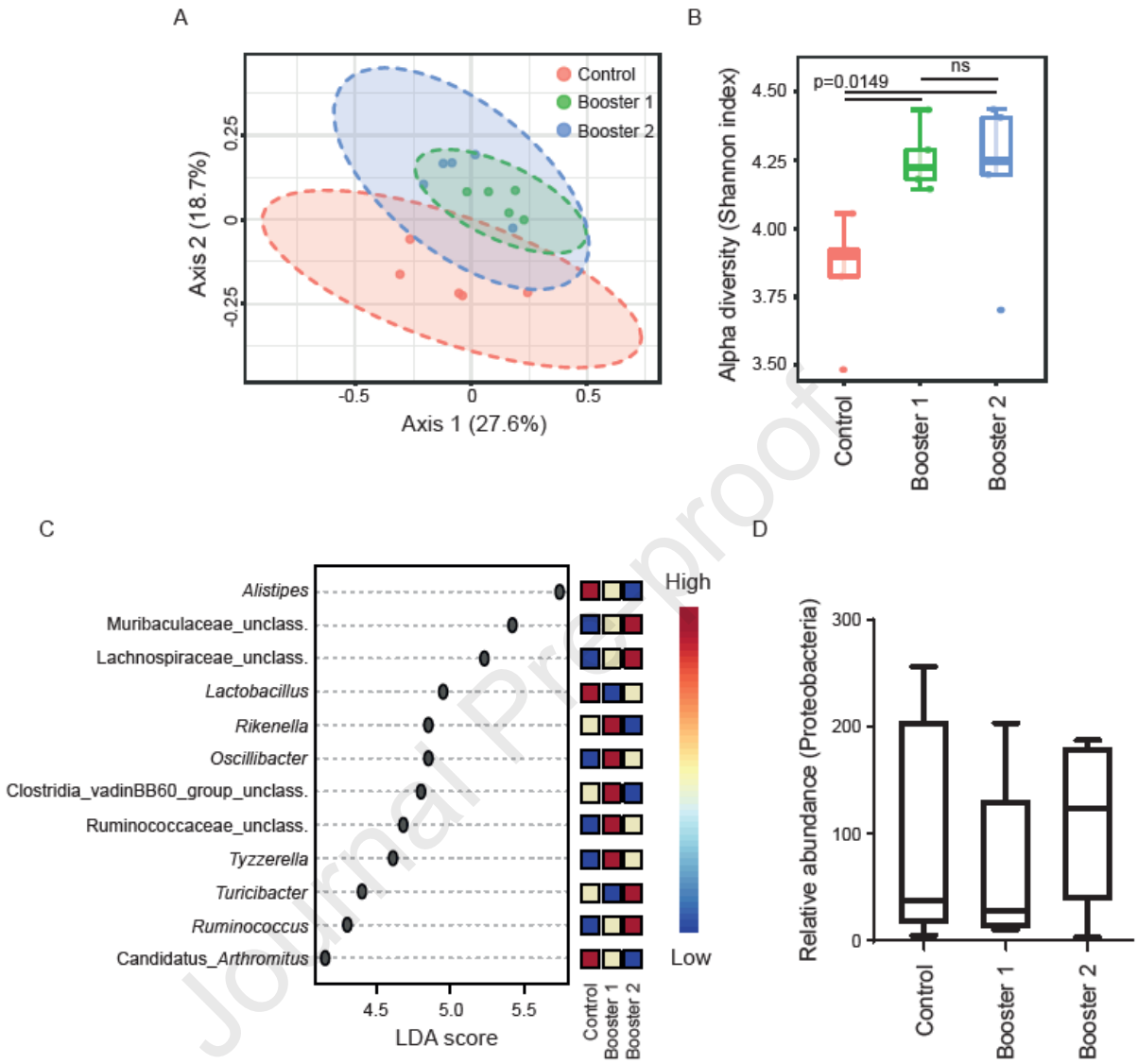

Figure 1: Cloning, expression and purification of rAbBamA. a) 1% agarose gel showing PCR amplification of *A. baumannii bam*A gene (lane 1). b) SDS-PAGE of rAbBamA expression demonstrating poor solubility (arrow). Lane 1: Soluble fraction before addition of IPTG (T0h); Lane 2: Insoluble fraction T0h; Lane 3: Soluble fraction after induction with IPTG for 4 hours (T4h); Lane 4: Insoluble fraction T4h. c) IMAC of rAbBamA. Lane 1: rAbBamA expressed after solubilization with 6M Urea; Lane 2: Flowthrough fraction; Lane 3: Eluted fraction 1; Lane 4: Eluted fraction 2.

Figure 2: a) Indirect ELISA demonstrating high antibody titers in anti-rAbBamA mice sera (mean $\pm$ SEM, n=5). b) Western blotting of rAbBamA and *A. baumannii* lysate proteins against pre-immune and anti-rAbBamA mice sera. Protein recognition can be observed (arrow). Lane 1: rAbBamA vs. Non-immunized mice sera pool; Lane 2: rAbBamA vs. Immunized mice sera pool; Lane 3: *A. baumannii* lysate vs. Non-immunized mice sera pool; Lane 4: *A. baumannii* lysate vs. rAbBamA Immunized mice sera pool.

Figure 3: Anti-rAbBamA binding on the surface of *A. baummannii*. a) Fluorescence microscopy of anti-rAbBamA mice serum in live, non-permeabilized *A. baummannii*, demonstrating antigenic recognition on the bacterial surface (arrows). b) Pre-immune serum was used as negative control. Scale bar represents 20µm, or 10µm in inset (white arrow).

Figure 4: Immunizations with rAbBamA lowered the bacterial burden on kidneys and increased survival of mice after intraperitoneal *A. baumannii* infection. a) Quantification of bacterial burden on kidneys (CFU/g) demonstrates a reduction of up to 4 CFU/g logs among the immunized group (n=10, Geometric mean ± SEM, **p<0.01). b) Mice monitoring for 7 days (n=10) after infection presents a 40% increase in animal survival of the immunized group when compared to the negative control (non-immunized).

Figure 5: Western blot of anti-rAbBamA sera against other Gram-negative bacteria membrane proteins. Protein bands with approximately 96 kDa (Lane 1: *A. baumannii*) and 30 kDa (Lanes 2 and 3, *E. coli* and *K. pneumoniae*, respectively). These 30kDa proteins were identified as OmpA and OmpC for both bacteria. No cross-reactivity was noted for *P. aeruginosa*.

Figure 6: Impact of immunization with rAbBamA on gut microbiota composition. a) Beta diversity of the gut microbiota of preimmune animals and animals that received one or two boosters, represented by Principal Coordinate Analysis of 16S rRNA gene sequencing data. Each dot represents one animal. b) Alpha diversity of gut microbiota represented by Shannon diversity indices. c) LEfSe analysis of gut microbiota composition at the genus level. d) Relative levels of sequences assigned to Proteobacteria. ns – not statistically significant.