



Article

Thiosulfinate-Enriched *Allium sativum* Extract as an Adjunct to Antibiotic Treatment of Sepsis in a Rat Peritonitis Model

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Featured Application: Thiosulfinate-enriched *Allium sativum* extract used as an adjuvant to antibiotic treatment and to sepsis management could improve the response profile and attenuate the outcome of the sepsis shock.

Abstract: Up to now, there are no studies that have shown a decrease in morbidity and mortality in the context of sepsis and septic shock, except for antibiotic therapy and the objective-guided resuscitation strategy. The goal was to evaluate the use of thiosulfinate-enriched *Allium sativum* extract (TASE) as an adjuvant in the management of sepsis. An experimental in vivo study was carried out with male Sprague Dawley® rats. Animals were randomized in three treatment groups: the control group (I), antibiotic (ceftriaxone) treatment group (II) and ceftriaxone plus TASE treatment group (III). All animals were housed and inoculated with 1×10^{10} CFU/15 mL of intraperitoneal *Escherichia coli* ATCC 25922. Subsequently, they received a daily treatment according to each group for 7 days. Clinical, analytical, microbiological, and histopathological parameters were evaluated. Statistically significant clinical improvement was observed in the ceftriaxone plus TASE vs. ceftriaxone group in weight, ocular secretions, whiskers separation and physical activity level ($p \leq 0.05$). When comparing interleukins on the third day of treatment between II and III, we found statistically significant differences in IL-1 levels ($p < 0.05$). Blood and peritoneal liquid cultures of group I were positive for multisensitive *E. coli*. Group II and III cultures were negative for *E. coli*, although an overgrowth of *Enterococcus faecalis* was found. In conclusion, TASE used as an adjuvant to antibiotic treatment in the management of sepsis could improve response profiles with sepsis attenuation, thus reducing overall mortality after an animal peritonitis model.

Keywords: garlic; *Allium sativum*; thiosulfinate; allicin; sepsis; immunomodulation; interleukins; rats

1. Introduction

There are few areas in critical medicine that generate as much interest and research as sepsis. Despite diagnostic and therapeutic advances, sepsis morbidity, mortality, and incidence remain very high. Sepsis is an altered host response to an infectious pathogen, causing potentially life-threatening organ dysfunction, and septic shock is a subset of sepsis in which the underlying circulatory and metabolic abnormalities are deep enough to substantially increase mortality [1]. Despite critical care progress in recent years in critical care, sepsis and septic shock account for more than 50% of deaths in critical care units.

Sepsis is now recognized as a multifactorial host response to an infectious pathogen that can be significantly amplified by endogenous factors involving the early activation of both pro- and anti-inflammatory responses along with major modifications in non-immunological pathways such as cardiovascular, neuronal, autonomic, hormonal, bioenergetic, metabolic and coagulative [2,3], all of which are of prognostic importance. Additionally, the biological and clinical heterogeneity of affected individuals is important, as well as age, underlying comorbidities, concurrent injuries (including surgery), medications, and source of infection [1].

What differentiates sepsis from infection is an aberrant or poorly regulated host response with the presence of organ dysfunction. The severity of organ dysfunction has been evaluated with various scoring systems that quantify abnormalities based on clinical findings, laboratory data, or therapeutic interventions. Differences in these scoring systems have also resulted in inconsistent information. The predominant score currently in use is the Sequential Organ Failure Assessment Score (SOFA), which has been simplified in quick SOFA [1,3].

Garlic (*Allium sativum*) has long been a medicinal ingredient used as an antineoplastic and antimicrobial agent. Sulfur compounds (i.e., thiosulfates) appear to be the active components in the root bulb of the garlic plant [4,5]. Allicin is the main thiosulfate of *Allium sativum* and could act on four points of the inflammatory cascade. The ability of allicin to inhibit the activation of the nuclear factor NF- κ B [6], prevent the adhesion of T cells to endothelial cells and reduce transendothelial migration [7] have been described. Allicin can also reduce the activity of induced nitric oxide synthase [8] and decrease the amount of nitric oxide and the vasodilatation that may lead to shock. Additionally, it could act by preventing the activation of the coagulation cascade by acting as an antiplatelet [9].

Up to now, there are no studies that have shown a decrease in morbidity and mortality in the context of sepsis and septic shock, except for antibiotic therapy and the objective-guided resuscitation strategy proposed in the 2016 sepsis campaign. This strategy was made by a group of international experts who established a series of based-on-evidence recommendations for the management of acute sepsis and septic shock. It is also the basis for the better outcome of high-mortality critically ill patients [10]. However, many drugs have been used unsuccessfully in both animal models and clinical trials [11–15], so there is still a need of new therapeutics that can overcome the antibiotic resistance.

As allicin is not stable [16], here, we decided to explore whether intraperitoneal applications of thiosulfate-enriched *Allium sativum* extract (TASE) could be an adjuvant to specific antibiotic treatment in sepsis and septic shock and to evaluate its possible immunomodulatory role.

2. Materials and Methods

2.1. Animals and Sepsis Model

Male 5-week-old Sprague Dawley[®] rats (Harlan Laboratories Models SL) were used. The study was conducted at the Translational Research Unit of the University General Hospital, Ciudad Real. The procedures were carried out at the same time of day to avoid the possible influence of the circadian cycle on the results of the work.

Rats were kept with food and water ad libitum, in a cycle of 12 h of light and 12 h of darkness, and a room temperature of 22 ± 2 °C with a relative humidity of 50–70% and 15–20 air renewals per hour without recirculation. They were housed according to RD

53/2013 and no rat was caged alone to favor their group behavior. In addition, they were maintained in these environmental conditions to allow acclimatization for a week before the study started. Animals were randomized in three groups. Group I: physiological saline ($n = 6$); group II: ceftriaxone ($n = 9$); and group III: ceftriaxone + TASE ($n = 9$). A model of peritonitis was generated in all groups. Rats from different groups were never housed in the same cage.

To create the peritonitis model, each rat was administered with an intraperitoneal injection of bacteria after anesthesia with ketamine/xylacin (75/10 mg/kg), also directly into the abdominal cavity. Prior to this experiment, we conducted an experimental study to determine the most optimal inoculum dose to generate the sepsis and septic shock model. We determined that it was necessary to use a concentration of *Escherichia coli* ATCC 25922 of 1×10^{10} colony forming units (CFU) in 15 mL of distilled water [17].

2.2. Thiosulfinate-Enriched *Allium sativum* Extract

Lyophilized *Allium sativum* extract was obtained from the purple garlic ecotype from Las Pedroñeras (Ciudad Real, Spain), the only European region with protected geographical status for garlic (ES/PGI/005/0228/12.03.2002). A patented protocol (WO 2008/102036 A1. Method for obtaining a freeze-dried, stable extract from plants of the *Allium* genus) was employed for extraction to guarantee the stability and concentration of allicin and other thiosulfates. The standardized composition and concentration of lyophilized *Allium sativum* extract are stable for over 10 months at 4 °C (Table 1, WO 2008/102036 A1). We employed diallyl thiosulfinate (allicin) concentration as the reference for the elaboration of the experimental treatment.

Table 1. Composition of organic and inorganic compounds of lyophilized *Allium sativum* extract from Las Pedroñeras (Ciudad Real, Spain) under optimized conditions (WO 2008/102036 A1).

Compound	Concentration (µg/mg)	Compound	Concentration (µg/mg)
Dimethyl thiosulfinate	18.30	Se	9.37
Allylmethyl+Methyl-allyl	4.58	B	89.45
Propyl-methyl+Methyl-propylthiosulfinate	3.39	Zn	10.60
Diallyl thiosulfinate (allicin)	5.62	Cd	9.48
Allyl-1-propenyl thiosulfinate	31.02	P	1188.87
1-propenyl-allyl+allyl-propyl thiosulfinate	1.76	Ca	159.11
Propyl-allyl thiosulfinate	1.59	K	3974.85
Di-propyl thiosulfinate	1.65	Mg	188.41
Methyl allyl sulfide	3.58	Cu	298.16
Methyl allyl disulfide	4.73	Fe	95.84
Dimethyl tetrasulfide	6.62	Cr	26.37
Di-allyl trisulfide	0.74	Si	3665.76
Di-methyl pentasulfide	1.63	Mn	1.18
Prostaglandin E1	4.83	Na	102.41
(E,Z)-Ajoene	0.07	Co	Non-detected
Inulin	0.10	Hg	Non-detected
Vitamin E (α-tocopherol)	3.07	Al, Ni	Non-detected

2.3. Experimental Design and Analytical Parameters

In relation to the treatments used, group I received 4.4 mL of 0.9% physiological saline intraperitoneally, group II received the same intraperitoneal volume with the antibiotic ceftriaxone (100 mg/kg) and group III the same volume with ceftriaxone (100 mg/kg) + TASE (0.5 mg/kg; referred to allicin content). In Figure 1, we showed the experimental scheme of the study.

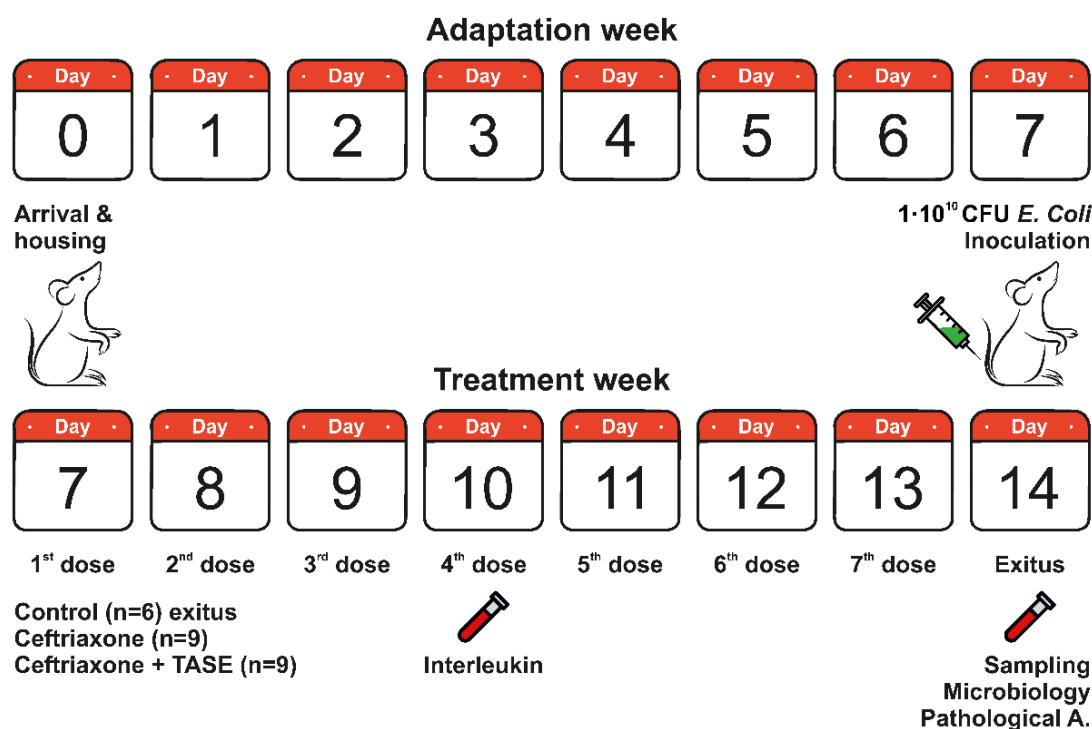


Figure 1. Chronological scheme of the study. Day zero started with the arrival of the animals, which included their respective housing, marking and the beginning of an adaptation process to the animal facility that lasted seven days. On the seventh day, *E. coli* inoculation and first treatment dose were performed. Blood samples for interleukin determination were taken 72 h after. On day 14, animals were sacrificed by lethal doses of anesthesia, with subsequent sampling for microbiology (blood and peritoneal fluid), interleukins (blood) and pathological anatomy (peritoneum, liver).

The following clinical parameters were evaluated daily: weight, mobility, appearance (normal, ocular secretions, nasal secretions, whisker position, lack of grooming, piloerection and dehydration), clinical signs (abdominal distension, hardening distension, temperature) and behavior (normal, hypoactive, lethargy; response to stimuli).

Interleukin (IL) 1 β /IL-1F2, IL-6 and tumor necrosis factor alpha (TNF- α) were determined in blood samples on treatment day 3 (T3) and 7 (T7) with the corresponding Quantikine[®] Rat ELISA method (R&D Systems), following the manufacturer's instructions. All samples were diluted 1:3 in RD5Y diluent. Each diluted sample and standards were processed in duplicate. Internal quality control was performed with recombinant buffered IL control material of known concentration. The final reading was made at 450 nm and corrected to 550 nm in a microplate reader.

Peritoneal fluid was also sampled on T7 (last day of the experiment) directly from the peritoneal cavity. Peritoneal fluid study was performed to determine cellularity by Giemsa staining. During the exploratory laparotomy, the degree of peritoneal inflammation was evaluated macroscopically. Liver and peritoneum samples were taken for histopathological evaluation. Thus, samples were paraformaldehyde fixed, paraffin embedded, and 4 μ m sections were made for hematoxylin/eosin staining to analyze the presence or absence of congestion and immune cells. A blinded expert pathologist evaluated the samples.

2.4. Statistical Analysis

A descriptive statistic of the quantitative variables was carried out to verify that the minimum and maximum values were in an adequate range. All data were expressed as mean \pm standard error of the mean ($\bar{x} \pm$ SEM).

To analyze the qualitative variables, proportions were compared with the chi-square test and Fisher's exact correlation. The means of the continuous variables were compared with U Mann–Whitney's test and normal distribution was verified by Shapiro Wilk's test.

In comparisons at different times within the same groups, tests were applied for paired variables (Student t for dependent variables or Wilcoxon test as the case may be). For the comparison between groups, the Kruskal–Wallis test was used as a function of normality. A significance level of 95% was used for statistical analysis. SPSS 25.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

Our model of abdominal sepsis is able to generate 100% lethality in rats if no antibiotic treatment is provided [17], so the control group could not finish the experimentation due to the fact that all members of the group died after 4–6 h post-inoculum. Unfortunately, one rat from group II also died after the inoculum despite the ceftriaxone administration, so we completed group II with eight rats.

In relation to clinical parameters, we found differences when comparing the body weights of group II and III on days 9, 10 and 11 ($p < 0.05$), corresponding to the third (T3), fourth (T4), and fifth (T5) dose of treatment (Figure 2). In relation to stress and suffering (nasal secretions, ocular secretions, whiskers position, lack of grooming, piloerection, lethargy, and diarrhea), we could only find differences in the level of activity of rats during the septic process 72 h post-inoculum (T3), showing greater hypoactivity in group II compared to group III ($p \leq 0.05$). Statistically significant differences were also observed in the position of the whiskers ($p \leq 0.05$) and in the presence of ocular secretions ($p \leq 0.05$) at that time point. No statistically significant differences were found in the rest of the clinical signs studied (Table 2), and from T4 to T7 (end of the study; data not shown).

In relation to the biochemical parameters studied (IL-1, IL-6, TNF- α), a comparison was made for the values of each interleukin between treated groups in T3 and T7 (Figure 3). Considering the levels of IL-1, in T3 we found statistically significant differences when comparing group II with respect to III ($p < 0.05$). It was also observed that IL-6 in T3 was lower in group III, although the values were not statistically significant. As for TNF- α , no differences between groups were assessed.

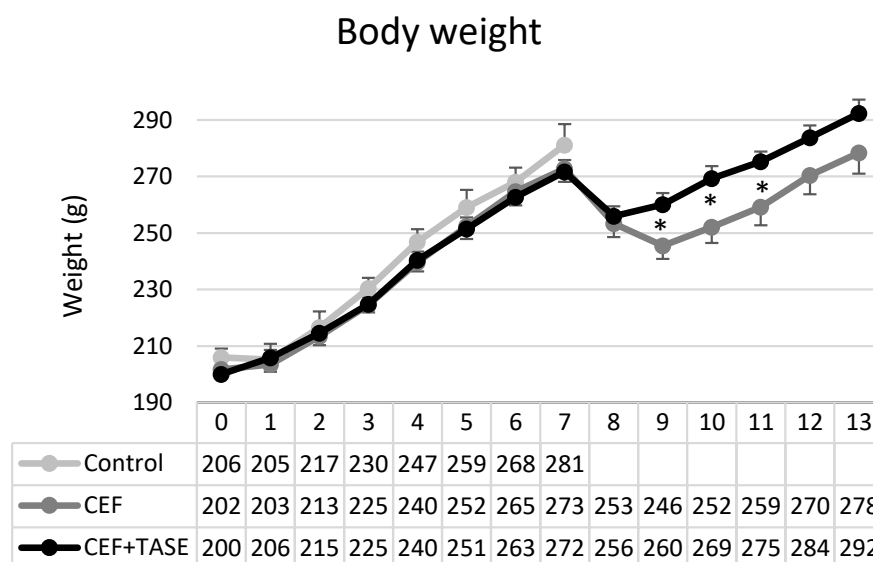


Figure 2. Weight monitoring during experiment: control group (8 days), group treated with ceftriaxone (CEF; 14 days) and group treated with ceftriaxone + thiosulfate-enriched *Allium sativum* extract (CEF + TASE; 14 days). On day 7, the bacterial inoculum was introduced. Mean \pm SEM. * $p < 0.05$.

Table 2. Clinical parameters in relation to stress and suffering at T1, T2 and T3 (24, 48 and 72 h post-inoculum, respectively) for nasal secretions, eye secretions, position of whiskers, lack of grooming, hair erection, lethargy, and diarrhea. * $p \leq 0.05$.

Variable, n/nt (%)	T1		p-Value
	CEF	CEF + TASE	
Nasal secretions 1	8/8 (100)	7/9 (78)	0.471
Ocular secretions 1	7/8 (88)	7/9 (78)	0.600
Whiskers separation 1	2/8 (25)	6/9 (67)	0.153
Lack of grooming 1	8/8 (100)	6/9 (67)	0.206
Piloerection 1	8/8 (100)	8/9 (89)	0.999
Hypoactivity 1	8/8 (100)	9/9 (100)	-
Diarrhea 1	2/8 (25)	0/9 (0)	0.206

Variable, n/nt (%)	T2		p-Value
	CEF	CEF + TASE	
Nasal secretions 2	7/8 (88)	6/9 (67)	0.576
Ocular secretions 2	6/8 (75)	5/9 (56)	0.620
Whiskers separation 2	2/8 (25)	7/9 (78)	0.057
Lack of grooming 2	8/8 (100)	6/9 (67)	0.206
Piloerection 2	7/8 (88)	5/9 (56)	0.294
Hypoactivity 2	6/8 (75)	4/9 (44)	0.335
Diarrhea 2	2/8 (25)	0/9 (0)	0.206

Variable, n/nt (%)	T3		p-Value
	CEF	CEF + TASE	
Nasal secretions 3	6/8 (75)	3/9 (33)	0.153
Ocular secretions 3	5/8 (63)	1/9 (11)	0.05 *
Whiskers separation 3	6/8 (75)	2/9 (22)	0.05 *
Lack of grooming 3	0/8 (0)	0/9 (0)	-
Piloerection 3	5/8 (63)	2/9 (22)	0.153
Hypoactivity 3	5/8 (63)	1/9 (11)	0.05 *
Diarrhea 3	1/8 (13)	0/9 (0)	0.471

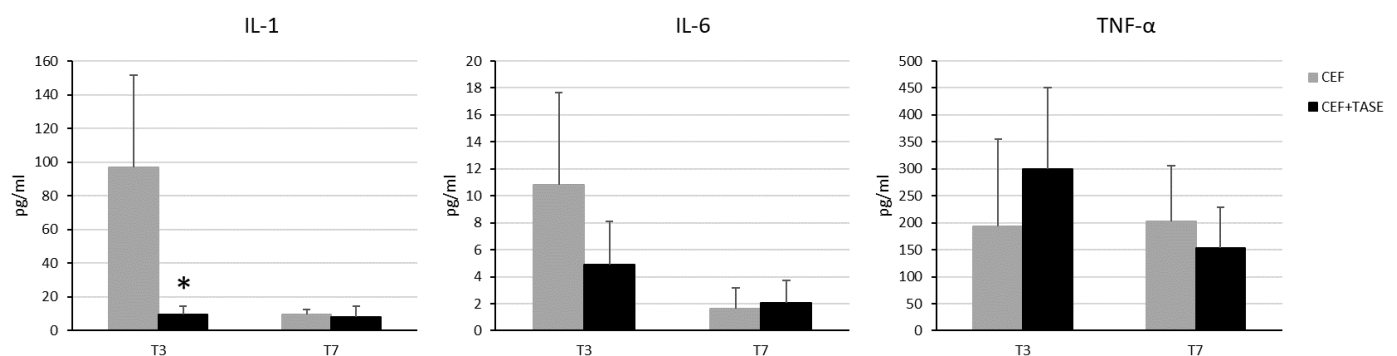


Figure 3. Interleukin levels in T3 and T7 (treatment day 3 and 7, respectively). CEF = ceftriaxone. CEF + TASE = ceftriaxone + thiosulfate-enriched *Allium sativum* extract. Mean \pm SEM. * $p < 0.05$.

The peritoneal liquid and blood cultures of the control group were positive for multi-sensitive *E. coli* ATCC 25922 and identical to the inoculum (Table 3). Additionally, as mentioned before, all the rats from the control group did not recover from the inoculum and died after 4–6 h, showing that our sepsis model is lethal if left untreated. In group II, only one rat died after inoculation, and of the remaining eight rats, six showed *Enterococcus faecalis* in blood cultures, and two in peritoneal liquid. In the blood cultures of the two remaining rats, the multi-sensitive bacteria *E. coli* ATCC 25922 appeared. In group III, *E. coli* ATCC 25922 was not detected neither in blood nor in peritoneal liquid. In fact, eight out of nine rats showed *Enterococcus faecalis* in blood samples, and only one out of nine rats

showed *Enterococcus faecalis* in peritoneal liquid. There was also one rat that was negative for both bacteria.

Table 3. Results of blood and peritoneal fluid cultures stratified by treatment groups and their respective antibiogram.

RESULTS OF MICROBIOLOGIC STUDIES				
	Number of Rats	Blood Cultures	Peritoneal Liquid Culture	Antibiogram (Sensitive to Ampicillin, Vancomycin, Teicoplanin)
Control (Group I)	3	Positive <i>E. coli</i> ATCC 25922	Positive <i>E. coli</i> ATCC 25922	Multisensitive
Ceftriaxone (Group II)	6	<i>Enterococcus faecalis</i>	2– <i>E. faecalis</i> 4–Negative	Sensitive
	2	Positive <i>E. coli</i> ATCC 25922	Negative	Multisensitive
	1	Exitus before 24 h (no samples collected)	-	-
Ceftriaxone + TASE * (Group III)	8	<i>E. faecalis</i>	1– <i>E. faecalis</i> 7–Negative	Sensitive
	1	Negative	Negative	-

* TASE (0.5 mg/kg; referred to allicin content).

Regarding the histopathological analysis and organ evaluation, the inflammatory cell count, the presence of bacteria in the liver and on the peritoneal surface, as well as the congestion and hepatic vacuolization between treatment groups (group II and III), no statistically significant differences were found (Table 4).

Table 4. Histopathological analysis and organs evaluation in relation to inflammation, bacteria, congestion, and vacuolization.

Variable, n/nt (%)	CEF	CEF + TASE	p-Value
Liver—hepatic congestion	8/9 (89)	8/9 (89)	-
Liver—sinusoidal PMN leukocytes	4/9 (44)	2/9 (22)	0.62
Liver—serosa PMN leukocytes	3/9 (33)	1/9 (11)	0.576
Liver—bacteria	2/9 (22)	0/9 (0)	0.471
Liver—perinuclear vacuolization	6/9 (33)	7/9 (78)	0.599
Peritoneum—PMN leukocytes	3/9 (33)	2/9 (22)	0.999
Peritoneum—bacteria	3/9 (33)	1/9 (11)	0.576

4. Discussion

Until now, many models of sepsis have been described in animal experimentation, but most of them failed to replicate the human heterogeneous septic process, which is dependent on the genetic susceptibility of each individual and influenced by sex, age, comorbidities and drug consumption [18,19]. The murine model described here generates an efficient, controlled and easily reproducible intraperitoneal infection that could serve as a basis for future lines of research.

Scientific research based on the use of garlic derivatives has led to ambiguous conclusions on the beneficial effects of this plant, thus preventing the application of garlic products in the treatment of certain diseases [20]. This situation can be attributed to several factors, among which the following can be highlighted: the chemical instability of this type of compound [21], the great diversity of industrial processes for their production, the lack of coherence in terms of the medical properties investigated, and the chemical composition of the products used in these clinical investigations [22]. With the freeze-dried

garlic used in this experimental model, these deficiencies could be overcome by using stable and known concentrations of allicin and other thiosulfonates over time.

At present, there are no studies in the scientific literature that have demonstrated a decrease in morbidity and mortality in relation to sepsis, except for antibiotics and the goal-guided resuscitation strategy [23]. Some of the drugs that have been tested are, among others, corticoids [12], immunoglobulins [13], antithrombin III [14], vasopressin [24] or anti-TNF [25]. However, the clinical results obtained in our animal experimentation study are encouraging in this sense and confirm our working hypothesis showing earlier recovery of weight, less ocular secretions, separation of whiskers and decrease in hypoactivity in the group where TASE was administered. We also found lower levels of IL-1 on the third day of treatment, and a tendency to decrease the pro-inflammatory cytokine IL-6 in the TASE group. All these data would support the immunomodulatory role of the lyophilized garlic, thanks to its action in the inflammatory cascade [26], thus achieving the attenuation of sepsis and septic shock. Previous work has described the ability to suppress inflammatory signals of lipopolysaccharide (LPS) through the expression of anti-inflammatory genes, and the reduction in pro-inflammatory cytokines (IL-6 and MCP-1) [27]. The work of Lee et al. [28] showed the immunomodulatory activity of garlic in an experimental sepsis model where clamping and blind puncture were performed to induce peritonitis. The authors described how the administration of methyl 3-formyl-4-methylpentanoate (a natural compound derived from garlic) led to the inhibition of apoptosis of lymphocytes in the spleen and significantly inhibited the production of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6. The production of TNF- α and IL-6 stimulated by LPS was also strongly inhibited by the compound methyl 3-formyl-4-methylpentanoate sucrose in macrophages derived from mouse bone marrow [28]. In our study, we could not assess any significant difference on TNF- α levels in the TASE group.

The microbiological analysis showed how the blood cultures of the control group were positive for *E. coli*. This bacterium corresponded to the same inoculum with which sepsis was generated. However, no blood culture or peritoneal fluid were positive for *E. coli* in the TASE-treated group. This result highlights the fact that the inoculum was sensitive to the antibiotic and adjuvant treatments used. In fact, it is known that there is a 90% sensitivity of *E. coli* to ceftriaxone and only 65% sensitivity of *Klebsiella pneumoniae*. Despite these moderately high percentages, we are currently in a global state of alarm due to the increased resistance of several microorganisms to this antibiotic compared to previous studies [29]. Moreover, most of the blood and peritoneal fluid cultures in our antibiotic treatment group showed the presence of *Enterococcus faecalis*. This result could be explained by considering the broad-spectrum efficacy of ceftriaxone on Gram-negative and some Gram-positive bacteria. This change of gastrointestinal microbiota would favor *Enterococcus faecalis*. One likely explanation for this selection is based on related studies in mice where LPS and flagellin from Gram-negative and anaerobic bacteria stimulated the production of RegIII γ in Paneth cells by interactions with Toll-like receptors. Paneth cells have an important role in the defense mechanisms of the gastrointestinal tract in several animal species, thanks to their secretions of lysozyme, phospholipase A2 and defensins [30]. RegIII γ is a C-type lectin receptor, capable of recognizing carbohydrates present on the surface of pathogens and is responsible of the internalization of the pathogen for antigen presentation and the induction of an immunological response. Thus, the level of RegIII γ maintains the balance between the bacteria that compose the intestinal microbiota and the host [31]. When ceftriaxone used in our study killed the Gram-negative bacteria, it decreased the production of RegIII γ and facilitated the growth of Gram-positive coccus (i.e., *Enterococcus faecalis*). Then, those Gram-positive could cross the intestinal barrier and reach the systemic circulation, liver and more. Therefore, if the antibiotic treatment persists for a long time, there could be a potential risk of bacterial endocarditis.

Few studies with animal models associate histological, clinical, and microbiological findings in intraperitoneal organs such as liver, peritoneum, and intestine after induced peritonitis. In this sense, our study tried to correlate those findings with the clinical response

to a treatment based on a thiosulfinate-enriched garlic extract. Only Lee et al. [28] examined the lung after peritonitis for these inflammatory changes, and also after therapy with a garlic derivative. Unfortunately, we could not obtain any histopathological difference between treatment groups because the tissue and organ evaluations were assessed at the end of the experiment and rats from both treatment groups were mostly recovered from the septic insult. Moreover, our study has several limitations that are inherent to the animal model of sepsis and septic shock that we use. First, the amount of blood collected was limited and did not allow the measurement of a greater number of inflammatory factors and biomarkers of endothelial damage. Secondly, no measurements were taken in relation to myocardial function and macrocirculation such as mean arterial pressure, contractility, peripheral vascular preload, and resistance to perform a target-guided therapy as it is usually performed in humans.

5. Conclusions

Thiosulfinate-enriched *Allium sativum* extract used as an adjuvant to antibiotic treatment and to sepsis management could improve the response profile and attenuate the outcome of the sepsis shock, mostly during the first days of the combined treatment. Further research would be necessary to clarify the immunomodulatory role of this plant extract.

6. Patents

Patent WO 2008/102036 A1. Method for obtaining a freeze-dried, stable extract from plants of the *Allium* genus.

National patent (Spanish Trademark number ES2675282A1). *Allium sativum* extract, its use for the manufacture of a medicinal product for the treatment of diseases, and its obtaining procedure.

Author Contributions: Conceptualization, F.J.R.-C., D.P.-V. and J.M.P.-O.; data curation, L.M.-P. and J.R.M.-R.; formal analysis, F.J.R.-C., O.M., V.B., N.V., S.I. and V.M.; investigation, O.M., N.B.-R., R.G. and L.M.-P.; methodology, D.P.-V., P.V. and S.I.; project administration, F.J.R.-C., D.P.-V. and J.M.P.-O.; resources, F.J.R.-C., D.P.-V. and L.A.G.; supervision, F.J.R.-C., D.P.-V. and J.M.P.-O.; visualization, J.R.M.-R.; writing—original draft, F.J.R.-C., O.M. and S.I.; writing—review and editing, F.J.R.-C., P.V., V.B., N.B.-R., R.G. and J.M.P.-O. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study (ref. PI-HGUCR 1/2014) was approved by the Animal Experimentation Committee of the University General Hospital, Ciudad Real. It was authorized by the Office of Agriculture of Castilla-La Mancha (Spain) and this experiment followed the ARRIVE guidelines developed by the National Center for the Replacement, Refinement and Reduction of Animals in Research (nc3rs).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Conflicts of Interest: L.A.G. is part of the authors of the registered brand Aliben© (European Trade mark number 10543429) which entitles the lyophilized *Allium sativum* extract employed in this study (patent WO 2008/102036 A1. Method for obtaining a freeze-dried, stable extract from plants of the *Allium* genus). D.P., P.V., J.M.P.-O., J.R.M.-R., L.A.G. and F.J.R.-C. are co-contributors of a national registered patent (Spanish Trade mark number ES2675282A1), which employs the lyophilized *Allium sativum* extract, its use for the manufacture of a medicinal product for the treatment of diseases, and its obtaining procedure.

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