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Amina EZZEROUG EZZRAIMI

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Étude de l'interaction bidirectionnelle entre les plaquettes et *Escherichia coli*

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Composition du jury

Pr. Jean-Philippe LAVIGNE	Rapporteur
Université de Montpellier	
Pr. Pascale GAUSSEM	Rapporteuse
Université de Paris	
Dr. Christian DEVAUX	Président du jury
Aix-Marseille Université	
Pr. Laurence CAMOIN-JAU	Directrice de thèse
Aix-Marseille Université	

Affidavit

Je soussignée, Amina EZZEROUG EZZRAIMI, déclare par la présente que le travail présenté dans ce manuscrit est mon propre travail, réalisé sous la direction scientifique de Professeur Laurence CAMOIN-JAU dans le respect des principes d'honnêteté, d'intégrité et de responsabilité inhérents à la mission de recherche. Les travaux de recherche et la rédaction de ce manuscrit ont été réalisés dans le respect à la fois de la charte nationale de déontologie des métiers de la recherche et de la charte d'Aix-Marseille Université relative à la lutte contre le plagiat.

Ce travail n'a pas été précédemment soumis en France ou à l'étranger dans une version identique ou similaire à un organisme examinateur.

Fait à Marseille, le 21 septembre 2022

Liste de publications et participation aux conférences

1) Liste des publications réalisées dans le cadre du projet de thèse :

1. Platelets and *Escherichia coli*: a complex interaction

Amina Ezzeroug Ezzraimi, Nadji Hannachi, Antoine Mariotti, Jean-Marc Rolain et Laurence Camoin-Jau (publié dans MDPI Biomedicines, https://doi.org/10.3390/biomedicines10071636)

2. The Antibacterial Effect of Platelets on *Escherichia coli* Strains

Amina Ezzeroug Ezzraimi, Nadji Hannachi, Antoine Mariotti, Clara Rolland, Anthony Levasseur, Sophie Alexandra Baron, Jean-Marc Rolain et Larence Camoin-Jau (publié dans MDPI Biomedicines, https://doi.org/10.3390/biomedicines10071533)

3. Microscopic description of platelet aggregates induced by *Escherichia coli* strains

Amina Ezzeroug Ezzraimi, Jean-Pierre Baudoin, Antoine Mariotti et Laurence Camoin-Jau (publié dans MDPI Cells https://www.mdpi.com/2073-4409/11/21/3495/htm)

4. Effect of antiplatelet agents on *Escherichia coli* sepsis: a review

Antoine Mariotti, **Amina Ezzeroug Ezzraimi** et Laurence Camoin-Jau (accepté dans Frontiers in Microbiolgy, Antimicrobials, Resistance and Chemotherapy)

5. Effect of antiplatelets on the interaction between blood platelets and *Escherichia coli* strains

Antoine Mariotti, **Amina Ezzeroug Ezzraimi**, Jean-Pierre Baudoin, Laurence Camoin-Jau (en cours de rédaction)

2) Participation aux conférences et écoles d'été au cours de la période de thèse :

Poster: The Antibacterial Effect of Platelets on *Escherichia coli* Strains
Journée Infectiopôle Sud, 01 juillet 2022- IHU Méditerranée Infection, Marseille, France.

Dédicace

Je dédié ce travail,

À mes parents ;

Ce diplôme ne sera pas le mien seulement, mais le NOTRE.

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Léon Tolstoï

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Résumé

Bien que le rôle des plaquettes dans les mécanismes de l'hémostase soit bien décrit, leurs fonctions pléiotropes sont moins documentées. Leur implication dans la réponse inflammatoire et anti-infectieuse est aujourd'hui démontrée et ouvre de nouvelles perspectives aussi bien dans la compréhension des mécanismes physiopathologiques que dans de nouvelles stratégies thérapeutiques. En effet, les plaquettes expriment des récepteurs qui peuvent interagir avec des bactéries. En fonction des espèces bactériennes, ces interactions vont induire une activation plaquettaire responsable du relargage du contenu des granules plaquettaires. Ces molécules présentes dans les granules plaquettaires possèdent des fonctions variées : bactéricides, pro-agrégantes, procoagulantes… De nombreux travaux se sont intéressés aux interactions entre les plaquettes et les bactéries Gram-positif. En revanche, peu de données sont disponibles sur les interactions entre les plaquettes et les bactéries Gram-négatif notamment *Escherichia coli.* De plus, les études disponibles présentent des résultats contradictoires.

Dans ce travail de thèse, nous nous sommes intéressés à l'étude de l'interaction bidirectionnelle entre les plaquettes et des souches d'*E. coli.* Notre revue de la littérature a eu pour objectif de faire l'état de l'art des connaissances actuelles sur les interactions entre les plaquettes et *E. coli* et leurs conséquences à la fois sur l'activité des plaquettes et sur la croissance bactérienne.

Le premier axe de nos travaux a évalué la capacité bactéricide des plaquettes sur dix souches d'*E. coli*. Nous avons sélectionné des souches de laboratoires et des souches cliniques afin d'évaluer leur capacité à induire *in vitro* une activation plaquettaire mais également leur sensibilité à l'activité bactéricide des plaquettes. Nous avons rapporté que cette interaction est souche dépendante. Elle pourrait être influencée également par la structure du lipopolysaccharide. De manière surprenante, nous avons observé que la croissance de certaines souches est augmentée en présence de plaquettes.

Afin de caractériser les interactions entre les plaquettes et les souches d'*E. coli,* nous avons utilisé la microscopie électronique. Cette approche innovante a confirmé que certaines souches induisaient une activation voire une agrégation plaquettaire. Les différences de structure des souches d'*E. coli*, pourraient expliquer ces différences dans les interactions plaquettes-bactéries.

Le second axe de notre travail de thèse a eu pour objectif de caractériser les mécanismes d'agrégation plaquettaire induite par *E. coli*. Le sepsis est, dans ses formes sévères, associé à des complications thrombotiques qualifiées d'immuno-thrombose, dans lesquelles les plaquettes jouent un rôle majeur. Nous avons fait une synthèse des connaissances actuellement disponibles sur les mécanismes d'agrégation plaquettaire au décours des sepsis à *E. coli* et une actualisation des effets des antiplaquettaires dans ces circonstances pathologiques.

En parallèle, nous avons caractérisé la capacité de dix souches d'*E. coli* à induire *in vitro* une agrégation plaquettaire. Ce mécanisme est comme l'activité bactéricide, souche-dépendant. Cette agrégation plaquettaire est réduite par un traitement antiplaquettaire sans cependant l'inhiber complètement. Ces résultats suggèrent que les voies de signalisation plaquettaire impliquées dans les mécanismes d'activation et d'agrégation plaquettaire sont multiples.

Mots clés : Plaquettes, *Escherichia coli*, activation plaquettaire, agrégation plaquettaire, antiplaquettaires

Abstract

Although the role of platelets in the mechanisms of hemostasis is well documented, their pleiotropic functions are the subject of numerous research works. Their role in the inflammatory and anti-infective response has now been demonstrated and opens new perspectives both in the understanding of pathophysiological mechanisms and in new therapeutic strategies. Indeed, platelets express receptors that can interact with bacteria. Depending on the bacterial species, these interactions will induce platelet activation responsible for the release the platelet granules content. These molecules present in the platelet granules have various activities: bactericidal, pro-aggregating, procoagulant…Many studies have focused on the interactions between platelets and Gram-positive bacteria. On the other hand, few data are available on the interactions between platelets and Gram-negative bacteria, in particular *Escherichia coli*. Moreover, the available studies present contradictory results.

In this thesis work, we were interested in studying the bidirectional interaction between platelets and strains of *E. coli*. Our review of the literature aimed to review the state of the art of current knowledge on the interactions between platelets and *E. coli* and their consequences on both platelet activity and bacterial growth.

The first axis of our work evaluated the bactericidal capacity of platelets on 10 strains of *E. coli*. We have selected laboratory strains and clinical strains in order to assess their ability to induce platelet activation *in vitro* but also their sensitivity to the bactericidal activity of platelets. We have reported that this interaction is strain-dependent. It could also be influenced by the structure of lipopolysaccharides. Surprisingly, we observed that the growth of certain strains is increased in the presence of platelets.

In order to characterize the interactions between platelets and *E. coli* strains, we used electron microscopy. This innovative approach has confirmed that certain strains induce platelet activation or even aggregation. The structural differences between strains of *E. coli*, could explain these differences in platelet-bacteria interactions.

The second axis of our thesis work aimed to characterize the mechanisms of platelet aggregation induced by *E. coli*. Sepsis is, in its severe forms, associated with thrombotic complications qualified as immuno-thrombosis, in which platelets play a major role. We have made a synthesis of the knowledge currently available on the mechanisms of platelet aggregation following *E. coli* sepsis and an update of the effects of antiplatelet drugs in these pathological circumstances.

In parallel, we characterized the ability of ten strains of *E. coli* to induce platelet aggregation *in vitro*. This mechanism is like bactericidal activity, strain-dependent. This platelet aggregation is reduced by treating the platelets with an antiplatelet molecule, however, without completely inhibiting it. These results suggest that the platelet signaling pathways involved in platelet activation and aggregation mechanisms are multiple.

Keys words: Platelets, *Escherichia coli*, platelet activation, platelet aggregation, antiplatelet drugs

Liste des abréviations

AAP : agents antiplaquettaires

ADP: adenosine diphosphate

CCL5 : CC-chemokine ligand 5

CD: Cluster of Differentiation

CIVD: coagulation intravasculaire disséminée

***E. coli*:** *Escherichia coli*

EHEC: entérohémorragique *Escherichia coli*

FcγRIIA: fragment crystallizable gamma Receptor

GPIIbIIIa: glycoprotéine IIbIIIa

HβD: human beta defensine

LPS : lipopolysaccharide

LPS-L : lipopolysaccharide-lisse

LPS-R : lipopolysaccharide-rugueux

ME : microscopie électronique

MEB : microscopie électronique à balayage

MET: microscopie électronique à transmission

MyD88 : différenciation myéloïde 88

PF-4: platelet factor-4

PMPs: peptides microbicides plaquettaires

SHU: syndrome hémolytique et urémique

TLR : toll like receptors

TNF : factor de nécrose tumorale

TxA2 : thromboxane A2

Tβ4: thymosin beta 4

Introduction générale

Les plaquettes sanguines ont été étudiées principalement pour leur rôle dans l'hémostase. Désormais, leur implication dans la réponse anti-infectieuse est confirmée (1–6). Les plaquettes peuvent interagir avec les bactéries par trois principaux mécanismes : i) la liaison aux bactéries par une protéine plasmatique qui est un ligand pour un récepteur plaquettaire, ii) la liaison directe des bactéries à un récepteur plaquettaire ou iii) la sécrétion de protéines bactériennes, c'est-à-dire de toxines, qui vont pouvoir interagir avec les plaquettes. Les mécanismes d'interaction dépendent de l'espèce bactérienne (2,6,7). Cette interaction peut engendrer une activation, une agrégation plaquettaire et le relargage de différentes molécules granulaires telles que les peptides antimicrobiens, qui possèdent une activité bactéricide, et des chimiokines qui vont être impliquées pour recruter d'autre cellules immunitaires (8–10). Cette capacité d'activation en réponse à une infection leur confère donc la possibilité de détruire les bactéries grâce à une activité bactéricide. Ceci démontre que leur activation et leur dégranulation jouent un rôle important dans la lutte anti-infectieuse (2,6,11).

La présence de mécanismes multiples dans ces interactions rend difficile l'identification des rôles des différentes protéines (tant bactériennes que plaquettaires). Cette analyse est encore plus compliquée par le fait que les interactions sont non seulement spécifiques de l'espèce mais aussi de la souche, comme le démontre notamment en 2016 Watson *et al* (12–14). Typiquement, les protéines bactériennes impliquées dans l'adhésion sont distinctes de celles qui induisent l'agrégation. Ainsi, les bactéries peuvent favoriser l'adhésion plaquettaire et/ou déclencher l'activation plaquettaire qui se caractérise par l'apparition ou l'augmentation de certains marqueurs de surface plaquettaires (P-sélectine (CD62P) et GpIIbIIIa (CD41) activée), ou par la sécrétion du contenu granulaire. Ces marqueurs sont le plus souvent détectés en cytométrie en flux, mais ne traduisent cependant pas forcément la formation d'un agrégat plaquettaire (15).

Les travaux effectués sur les interactions plaquettes-bactéries, ont permis d'avoir des connaissances sur l'interaction des plaquettes avec les bactéries Gram-positif (3,16–19). En revanche, peu de données sont disponibles sur les bactéries à Gram-négatif à savoir *Escherichia coli* (*E. coli*), notamment sur les mécanismes moléculaires d'interaction et le pouvoir inhibiteur des molécules plaquettaire sur ces bactéries.

E. coli est une bactérie Gram-négatif commensale, appartenant à la famille des entérobactéries, retrouvée très fréquemment dans le tractus digestif de l'être humain, et représentant une grande partie de la flore intestinale. Certaines souches sont souvent retrouvées

en pathologie humaine, notamment dans les infections communautaires et nosocomiales, dans des sites très variées : méningites, gastro-entérites, infections urinaires… (20,21).

La plupart des travaux étudient l'effet des pathovars d'*E. coli* responsables de syndrome hémolytique et urémique (SHU) sur les plaquettes. En effet, le SHU, qui se caractérise notamment par une anémie hémolytique d'origine mécanique et une thrombopénie, a fait l'objet de nombreuses études. Une activation plaquettaire est observée, conséquence de l'activation de l'endothélium par la production de Shiga toxines (22). Cependant, les mécanismes et les conséquences des interactions entre les souches *E. coli* responsables du SHU et les plaquettes sont complexes et variables selon les souches. La souche d'*E. coli* O111 pourrait interagir directement avec les plaquettes par l'intermédiaire du récepteur toll like-4 (TLR-4), entrainant une augmentation des marqueurs d'activation plaquettaire et l'expression du facteur tissulaire (23). En revanche, la souche d'*E. coli* O157 :H7, induit une agrégation plaquettaire médiée par récepteur FcγRIIA indépendamment du TLR-4 (24).

Des souches d'*E. coli* ou leurs lipopolysaccharide (LPS) purs sont capables d'induire une activation plaquettaire avec l'implication des récepteurs plaquettaires comme le TLR-4 et le FcγRIIA. Cependant, cette réponse est hautement variable. Elle peut dépendre de plusieurs facteurs tels que la souche étudiée, la préparation des plaquettes utilisée et le ratio plaquettes-bactéries (23–27).

E. coli peut également induire une agrégation plaquettaire dépendante à la fois de la souche impliquée, ainsi que du ratio plaquettes/bactéries présent. La concentration de l'inoculum joue aussi un rôle important. Ces interactions reposent principalement sur le FcγRIIA et l'intégrine αIIbβ3 (12,26).

Enfin, la structure du LPS serait également un élément déterminant dans ces interactions. Certaines études n'ont utilisé que le LPS pur pour évaluer son effet sur les plaquettes et les résultats montrent qu'il induit une réponse variable impliquant des récepteurs plaquettaires différents (25–27). Cette différence peut être expliquée par le fait qu'en fonction de l'antigène-O, les souches d'*E. coli* possèdent deux formes du LPS, rugueux (LPS-R) ou lisse (LPS-L). Ainsi, cette capacité ou non à faire agréger les plaquettes pourrait donc dépendre de la structure des LPS et de leur capacité ou non à être reconnus par le TLR-4 exprimé à la surface des plaquettes. Ainsi, l'antigène-O pourrait également avoir un rôle important dans la reconnaissance de ces motifs bactériens par les plaquettes et dans l'induction ou non d'un choc septique (28).

Les plaquettes ont un impact paradoxal lors d'une infection bactérienne. Elles présentent un effet antimicrobien direct, des capacités de réparation tissulaire et elles permettent une

immunomodulation de la réponse immunitaire ainsi qu'un certain chimiotactisme. Cependant, leur activation non contrôlée et disséminée peut conduire à l'aggravation du sepsis, qui peut induire une coagulation intravasculaire disséminée (CIVD), responsable d'un risque thrombotique mais aussi hémorragique. Ces observations nous conduisent à réfléchir à l'utilisation des agents antiplaquettaires (AAP) au cours de sepsis qui diminuerait la réactivité plaquettaire délétère au risque d'inhiber la réponse anti-infectieuse bénéfique des plaquettes. En effet, il est important de maintenir un équilibre entre les effets bénéfiques et délétères des plaquettes au cours des infections, notamment le sepsis (29).

Nos travaux de thèse se sont intéressés aux interactions bidirectionnelles entre les plaquettes et des souches d'*E. coli*. En effet, nous avons étudié en parallèle les conséquences de ces interactions sur les fonctions plaquettaires et sur la croissance bactérienne.

Dans un premier temps, notre revue de la littérature présente les connaissances actuelles sur les interactions entre les plaquettes et *E. coli.* Notre objectif était de réaliser une synthèse des données obtenues à partir des études expérimentales et cliniques sur l'action d'*E. coli* sur l'activation plaquettaire d'une part, mais également et sur l'effet des plaquettes sur les souches d'*E. coli* **(Article 1).**

Nos travaux s'organisent autour de deux axes principaux. Le premier axe a consisté à étudier la capacité bactéricide des plaquettes sur la croissance de différentes souches d'*E. coli.* Nous avons réalisé cette étude sur un panel de dix souches ; cinq d'entre elles provenaient d' isolats cliniques et cinq étaient des souches de laboratoire. Ces dix souches présentaient des profils de sensibilité ou de résistance à la colistine différents. Cet axe a nécessité la maitrise de plusieurs méthodologies, notamment la coculture des bactéries et les plaquettes pour évaluer l'effet des plaquettes sur la croissance bactérienne, la cytométrie en flux pour mesurer l'expression du marqueur d'activation plaquettaire, l'analyse du pangénome et enfin la microscopie électronique à balayage et à transmission pour visualiser les agrégats plaquettaires induites par *E. coli* (Figure 1).

En évaluant l'interaction bidirectionnelle entre les plaquettes et *E. coli*, nous avons démontré une variabilité de réponse des souches. Certaines souches peuvent induire une activation plaquettaire. A l'inverse, certaines souches n'induisent aucune d'activation plaquettaire. Nous avons également démontré que l'activité bactéricide des plaquettes est liée au niveau d'activation, ce qui est corrélé avec les résultats de microscopie. L'aspect des agrégats, l'état de l'activation ainsi que l'abondance et la localisation des bactéries nous ont confirmé que les conséquences de ces interactions sont dépendantes de la souche testée et de sa capacité à induire une activation, voire une agrégation plaquettaire. Pour expliquer cette

hétérogénéité de réponse, nous avons sélectionné deux souches apparentées et qui ont des profils opposés vis-à-vis des plaquettes. Nos résultats suggèrent la présence ou absence de l'antigène-O au niveau du LPS pourrait être un facteur de variabilité. Cette hypothèse est appuyée par le fait que les plaquettes peuvent interagir avec *E. coli* à travers le couple TLR4-LPS **(Article 2 et 3)**.

Dans le second axe, nous nous sommes intéressés à la capacité des souches d'*E. coli* à induire une agrégation plaquettaire et à l'action de molécules antiplaquettaires sur ce mécanisme. Nous avons rédigé une revue sur les connaissances actuellement disponibles sur les mécanismes d'agrégation plaquettaire induits par *E coli*, le sepsis à *E. coli*, ainsi qu'une actualisation des effets des antiplaquettaires dans ces circonstances pathologiques **(Article 4)**.

L'article 5 avait pour objectif d'étudier l'agrégation plaquettaire induite par des souches d'*E. coli*. Dans cette étude, les dix souches d'*E. coli* précédemment décrites. Nous avons évalué la capacité de ces dix souches à induire l'agrégation plaquettaire par deux méthodes complémentaires : la spectrophotométrie et la microscopie confocale. L'effet des médicaments antiplaquettaires a été évalué sur les souches responsables de l'agrégation plaquettaire. Nos résultats montrent que l'agrégation induite par *E. coli* est dépendante de la souche testée mais aussi de la concentration utilisée. Cette variabilité s'illustre également en microscopie confocale. Certaines souches sont capables d'induire une agrégation donc elles se trouvent à l'intérieur des amas plaquettaires. D'autres souches n'ont pas d'effet sur l'agrégation et les bactéries se trouvent en proximité des plaquettes.

Les médicaments antiplaquettaires testés permettent de réduire l'agrégation plaquettaire observée induite par certaines souches sans l'inhiber complètement **(Article 5)**.

L'ensemble de ces travaux présentent une contribution significative permettant une meilleure compréhension des mécanismes complexes des interactions entre les différentes souches d'*E. coli* et les plaquettes.

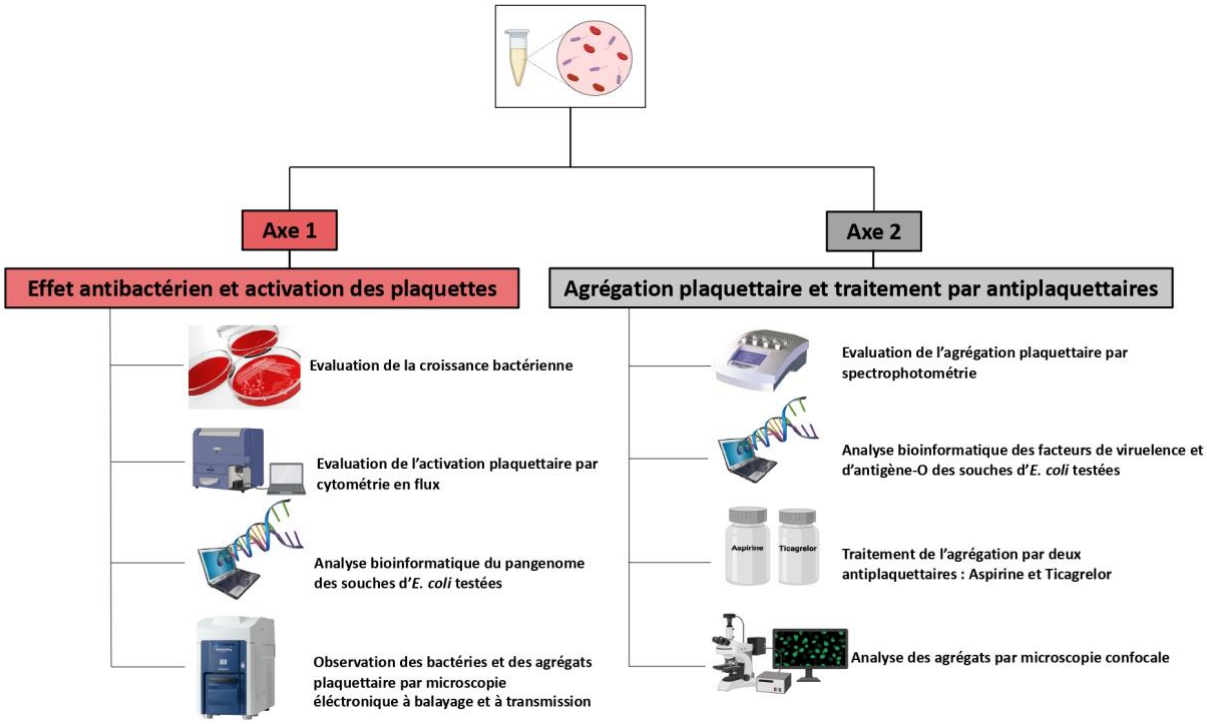

Figure 1 : Schéma récapitulatif des techniques utilisées dans nos travaux de thèse.

Chapitre I : Revue de la littérature

Préambule

Bien que de nombreuses études se soient intéressées à l'interaction entre les plaquettes et les bactéries, peu de données sont disponibles sur l'*E. coli.* L'objectif de cette revue, est de faire une synthèse des études expérimentales disponibles sur la relation bidirectionnelle entre les plaquettes et *E. coli.*

Nous nous sommes intéressés principalement à l'activation et l'agrégation plaquettaire induites par *E. coli*, ainsi qu'à la capacité des plaquettes à inhiber la croissance des bactéries.

Afin de comprendre les mécanismes d'interaction, nous avons analysé les données des études faites sur les principaux pathovars d'*E. coli,* y compris les pathovars entérohémorragiques (EHEC) qui sont impliqués dans le SHU et qui secrètent la Shiga toxine ainsi que d'autre souches cliniques et de laboratoire. Notre analyse met en évidence le rôle majeur de deux récepteurs plaquettaires : TLR-4 et FcγRIIA. Ces éléments jouent un rôle clé dans la reconnaissance, la liaison et l'activation des plaquettes.

Les résultats de cette analyse bibliographique, ont montré que l'interaction plaquettes-*E. coli* est à double sens (30–35). Les conséquences des interactions entre les plaquettes et les différentes souches d'*E. coli* induisent des effets très variables sur les fonctions plaquettaires. Plusieurs facteurs peuvent influencer les réponses, tels que la souche ou le LPS testés, la préparation des plaquettes, le ratio plaquettes-bactéries… La différence de forme du LPS, plus précisément la structure de l'antigène-O, qui est d'ailleurs utilisée pour le sérotypage des souches d'*E. coli,* permettrait d'expliquer ces résultats.

Enfin, en tenant compte des similitudes de structure et de polarité entre les peptides plaquettaires cationiques et la colistine (polymyxine E), nous avons noté qu'il est important de prendre la résistance aux antibiotiques en considération, qui pourrait également être un facteur de variabilité.

L'effet d'*E. coli* sur les plaquettes doit être étudié, en utilisant un panel plus large de souches d'*E. coli* afin de comprendre au niveau moléculaire les mécanismes d'activation, d'agrégation et de libération des molécules antimicrobiennes, d'autant plus qu'il existe des résultats contradictoires même dans les études réalisées sur une souche identique (26,27,36). L'implication de multiples récepteurs plaquettaires doit également être mise en évidence afin de pouvoir cibler et inhiber les facteurs conduisant au développement des pathologies générées par les souches de *E. coli*.

Article 1 : Platelets and *Escherichia coli*: A Complex Interaction.

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Review

Platelets and *Escherichia coli*: A Complex Interaction

Amina Ezzeroug Ezzraimi [1,2], Nadji Hannachi [1,3], Antoine Mariotti [1,2,4], Jean-Marc Rolain [1,2] and Laurence Camoin-Jau [1,2,4,*]

1 IRD, APHM, MEPHI, IHU Méditerranée Infection, Aix Marseille Université, 19-21 Boulevard Jean Moulin, 13005 Marseille, France; amina.ezzeroug.ezzraimi@gmail.com (A.E.E.); n_adji07@live.fr (N.H.); antoine-julien.mariotti@ap-hm.fr (A.M.); jean-marc.rolain@univ-amu.fr (J.-M.R.)
2 IHU Méditerranée Infection, Boulevard Jean Moulin, 13385 Marseille, France
3 Département de Pharmacie, Faculté de Médecine, Université Ferhat Abbas Sétif I, Sétif 19000, Algeria
4 Hematology Department, Timone Hospital, APHM, Boulevard Jean Moulin, 13005 Marseille, France
* Correspondence: laurence.camoin@ap-hm.fr; Tel.: +33-4-9138-6049; Fax: +33-4-9138-9155

Abstract: Apart from their involvement in hemostasis, platelets have been recognized for their contribution to inflammation and defense against microbial agents. The interaction between platelets and bacteria has been well studied in the model of *Staphylococcus* and *Streptococcus* but little described in Gram-negative bacteria, especially *Escherichia coli*. Being involved in the hemolytic uremic syndrome as well as sepsis, it is important to study the mechanisms of interaction between platelets and *E. coli*. Results of the published studies are heterogeneous. It appears that some strains interact with platelets through the toll-like receptor-4 (TLR-4) and others through the Fc gamma glycoprotein. *E. coli* mainly uses lipopolysaccharide (LPS) to activate platelets and cause the release of antibacterial molecules, but this is not the case for all strains. In this review, we describe the different mechanisms developed in previous studies, focusing on this heterogeneity of responses that may depend on several factors; mainly, the strain studied, the structure of the LPS and the platelet form used in the studies. We can hypothesize that the structure of O-antigen and an eventual resistance to antibiotics might explain this difference.

Keywords: *Escherichia coli*; platelets; LPS; PMP

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1. Introduction

If platelets were exclusively associated with hemostasis and wound healing for a long time, it is now recognized that they play a key role in the fight against infection. Indeed, platelets share a structure and functional characteristics with immune cells, especially of the myeloid lineage. On their outer membranes, platelets express a set of receptors that allow the recognition of pathogens, making them immune sentinels [1]. During the activation process, platelets quickly change from quiescent discoid forms to amoeboid cells that move to sites of infection.

Studies have shown that a significant number of bacterial species responsible for infections in humans can interact with platelets, causing differences in platelet activation and aggregation. It has been reported that *Staphylococcus aureus* and *Streptococcus pyogenes* rapidly attached to and aggregated on human platelets in vitro [2,3], whereas *Escherichia coli* and *Enterococcus faecalis* caused much slower platelet aggregation. In vitro studies have shown that some strains of bacterial species such as *Fusobacterium, Listeria, Mycobacterium, Pseudomonas, Salmonella* and *Yersinia* are able to induce platelet activation and aggregation [4]. However, the effects obtained depend on the bacterial strain, the platelet-to-bacteria ratio and the inter-individual platelet variability. Differences in this interaction lead to differences in the platelet activation and aggregation process, which likely influences host defense against pathogens. In general, the events associated with the interaction between platelets and bacteria proceed through distinct and progressive

phases: direct contact, morphogenesis from discoid to amoeboid form, initial aggregation, and irreversible aggregation. Platelets can be activated by direct contact between platelet receptors and bacterial surface protein, and also by bacterial secreted molecules such as toxins, or indirectly activated via a plasma protein which acts as a bridge between the platelet and the bacteria [3], thus involving platelet receptors FcγRIIA, glycoprotein (GP) αIIbβ3, GPIbα, toll-like receptors, and complement [5].

In response to an activating stimulus, such as bacteria, platelets release molecules which, in addition to their role in the hemostatic process, have been shown to have an antimicrobial effect, and which are today grouped under the name of platelet microbicidal peptides (PMPs) [4].

The mechanisms of interaction between platelets and bacteria have been widely described as in the case of *Staphylococcus aureus* [2,6,7]. On the other hand, they are less detailed in Gram-negative, especially *E. coli*. The objective of this review is to summarize the available data on the mechanisms of interaction between *E. coli* and platelets and their consequences.

2. *Escherichia coli* Pathovars

E. coli is a commensal bacterium of the gastrointestinal tract of humans, other mammals and birds as well as cold-blooded animals. There are several pathogenic strains which can cause clinical syndromes such as diarrhea, enteric infections, urinary tract infections, meningitis and septicemia, which represent significant clinical cases and cause thousands of deaths per year. The pathogenicity of strains can be explained by different virulence mechanisms such as adhesin expression, toxin secretion, iron acquisition factors, lipopolysaccharide structure, presence of polysaccharide capsules and invasin expression [8,9]. Based on these characteristics, pathogenic *E. coli* can be classified into two groups (Figure 1): intestinal pathogenic *E. coli* (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC). Each of these two groups contains different subgroups, causing various problems [10].

Despite the ability of extraintestinal pathogenic *E. coli* (ExPEC) group strains to be associated with infections in the intestinal tract, their virulence is significantly higher when they colonize tissues outside the intestine [11,12]. ExPEC themselves can be grouped into five categories; uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC), neonatal meningitis-associated *E. coli* (NMEC), avian pathogenic *E. coli* (APEC) and *E. coli* mammary pathogenic *E. coli* (MPEC) [8,13].

As for intestinal pathogenic *E. coli* (InPEC), they can be grouped into seven categories: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), entero-invasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), adherent invasive *E. coli* (AIEC) and diffuse adherent *E. coli* (DAEC) [8,13].

EHEC strains are the most studied, due to their ability to strongly colonize the mucosa of the distal ileum and the colon and to induce so-called "attachment and effacement" lesions of enterocytes via a protein (intimin) encoded by the *eae* gene [14,15]. They are characterized by their somatic antigen O and their flagellar antigen H. The serotypes most involved in epidemics belong to serotypes O26: H11, O103: H2, O111: H8, O145: H28 and O157: H7 [16,17]. EHEC group can cause mild watery diarrhea and hemorrhagic colitis that can progress to severe forms, such as hemolytic-uremic syndrome (HUS), mainly in young children, or thrombotic microangiopathy (TMA) in adults. EHEC, more specifically the Shiga toxin-producing strain *E. coli* STEC, can also release toxins such as Shiga toxins (Stx), inducing damage to the vascular endothelium, mainly intestinal, renal and cerebral, whose *stx* genes have been transferred by bacteriophages [18].

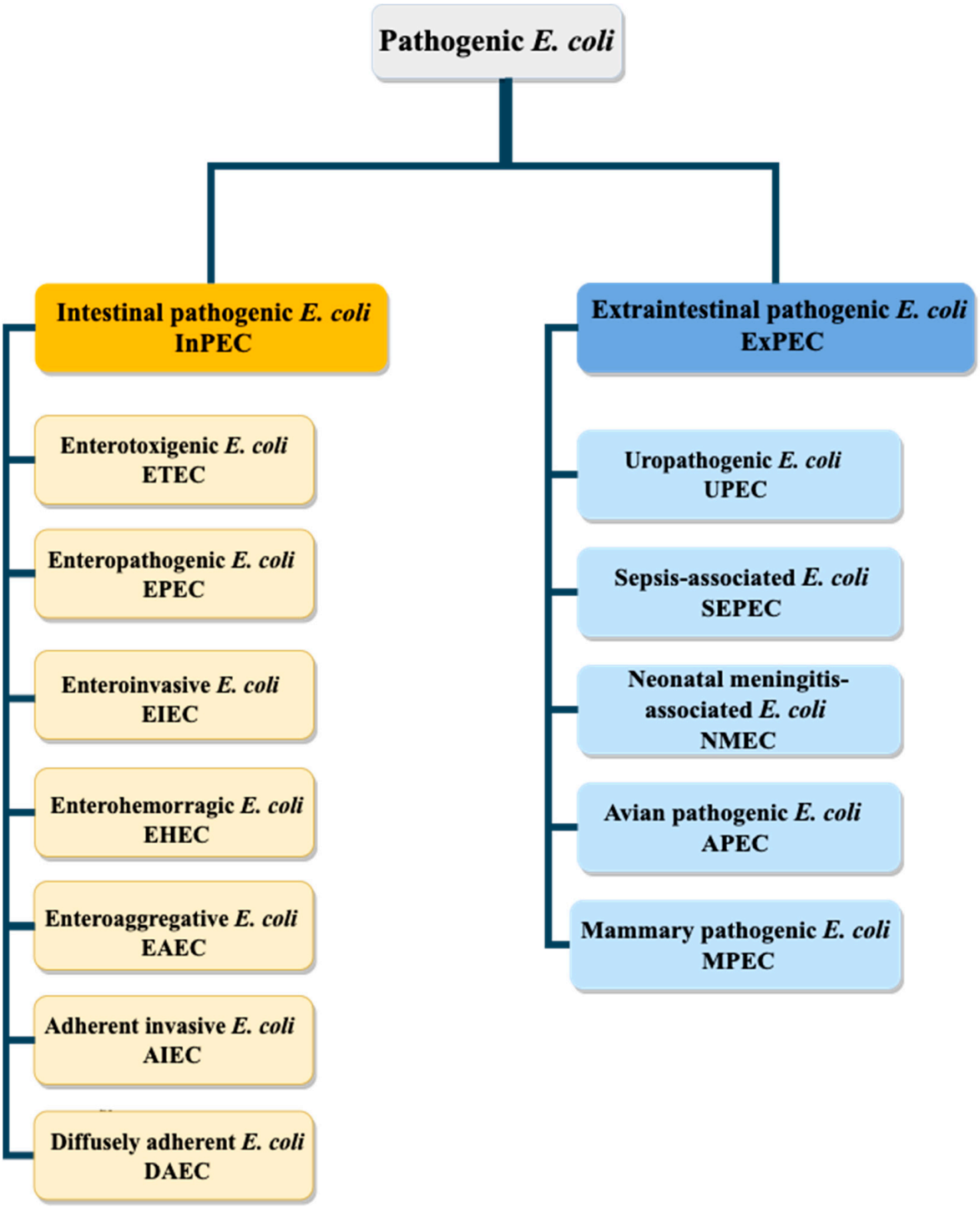

Figure 1. Diagram of pathogenic *E. coli* strains. *E. coli* pathogenic strains can be divided into two groups: extraintestinal pathogenic *E. coli* (ExPEC) and intraintestinal pathogenic *E. coli* (InPEC).

3. Mechanism of Interaction between Platelets and *E. coli*

3.1. Platelet Receptors

In this part we describe two main platelet receptors: the toll-like receptors (TLRs) and Fc receptors that are involved in interactions with *E coli.*

TLRs belong to a family of receptors that recognize molecular pattern recognition molecules associated with pathogens. The structure of TLRs is characteristic of type I membrane proteins with a transmembrane domain which spans the membrane and

connects to an extracellular domain [19]. TLRs play a crucial role in the early innate immune response to pathogens, as the primary defense system against microbial infection.

The interaction of TLRs with microbial products leads to activation of multiple intracellular signaling pathways, which are generally through a MyD88-dependent pathway leading to the stimulation and secretion of inflammatory cytokines, and a Toll/IL-1R domain-containing adaptor inducing IFN-beta (TRIF)-dependent pathway that causes Interferon-β stimulation and dendritic cell maturation [20].

Platelets express TLRs; this highlights the role of platelets in the innate immune response. TLRs 1, 2 and 6 are moderately expressed [21–23]. The role of TLR2 has been studied in the activation of the platelet response to *Streptococcus pneumoniae*, which induces platelet aggregation and adenosine diphosphate (ADP) release in both a TLR2- and a TLR4-dependent manner [24,25]. TLR3 is very weakly expressed at the cell surface and intracellular level. Agonizing TLR3-platelet receptors would induce heterogeneous effects [26,27]. TLR7, TLR5, 8 and 10 represent the least studied receptors [28].

Regarding *E. coli*, TLR4 represents the most studied platelet receptor involved in the interaction with this bacterium. The presence of TLR4 was first identified on mouse and human platelets using flow cytometry [29]. As we are specifically interested in the interaction between platelets and *E. coli*, we focused on the TLR4 which is involved in this interaction. LPS stimulates platelet secretion and aggregation requiring the TLR4/MyD88 complex [30,31]. Andonegui et al. were the first to demonstrate that *E. coli* LPS-induced thrombocytopenia was dependent on platelet TLR4. Indeed, thrombocytopenia was observed after administration of LPS only in wild-type mice, not in TLR4-deficient mice. Similarly, an accumulation of platelets in the lungs was observed only in wild-type mice [29]. TLR4 also plays a role in TNF-alpha production after LPS administration in a mouse model. This clearly highlights the role of TLR4 in the process of LPS-induced thrombocytopenia. However, the mechanism by which platelets contribute to increased TNF-alpha levels is still unclear [32].

The strain and concentration of LPS used in these in vitro models are also important variables to consider when analyzing platelet activation by LPS. Human platelets may respond differently to various bacterial LPS via TLR4 engagement, resulting in distinct cytokine secretory profiles with different potency depending on the strains triggering platelet responses [33].

Apart from TLR class receptors, FcγRIIA also plays a key role in *E. coli* and platelet interactions. It is a Type I transmembrane protein of approximately 40 kDa and consists of two extracellular Ig-like domains, a single transmembrane domain and a cytoplasmic tail that bears an immunoreceptor tyrosine-based activation motif (ITAM) domain with dual YXXL amino acid consensus sequences. These receptors are found in platelets, neutrophils and monocytes [34].

Human platelets represent the richest source of FcγRIIA in the body, containing 1000 to 4000 copies of this type of receptor [35]. Platelets, through the FcγRIIA can coat opsonized entities such as bacteria, which leads to platelet activation and the secretion of multiple molecules in order to respond against pathogens [5]. It is through the second Ig-like domain that immunoglobulins bind to FcγRIIA [34]. FcγRIIA is also implicated in signaling being an adapter protein for the platelet integrin αIIbβ3 [34]. Arman et al. have shown that, in addition to the role of FcγRIIA in the initiation of platelet activation after having been involved in the oppsonization of bacteria, the secretion of alpha and dense granules dependent on FcγRIIA and integrin alpha IIb-beta 3 (αIIbβ3) is also important for triggering bacterial aggregation [36]. αIIbβ3, in turn, is also able to stimulate platelet aggregation after being bound to soluble fibrinogen [34]. Regarding *E. coli*, the role of FcγRIIA in platelet activation and aggregation (Table 1A) is linked to conflicting results that will be further detailed below.

3.2. E. coli Products

LPS is an important structural component of the outer membrane of Gram-negative bacteria and one of the most studied immunostimulatory elements of bacteria. They can induce systemic inflammation and sepsis if excessive signals occur. LPS consists of three main components: lipid A, the core oligosaccharide which can be divided into an external and an inner part, and thus the O-antigen polysaccharide. Two types of LPS are distinct: smooth and rough LPS. Smooth LPS (S-LPS) contains an O-antigen polysaccharide, additionally to lipid A and core oligosaccharide. In contrast, rough LPS (R-LPS) is deficient in an O-antigen. It has been found that numerous clinical Gram-negative bacteria have S-LPS [37]. LPS can interact with host proteins, including LPS-binding protein, CD14, MD-2 and TLR4 [38]. Platelet activation stimulated by LPS also includes the triggering of pro-inflammatory response, such as the production of cytokine Inteleukin-1 beta, which in turn activates endothelial cells and platelets and participates in tissue damage related to bacterial invasion [27].

Studies have focused on the interaction between LPS from EHEC strains and platelets, with contradictory results. Indeed, some of them have shown that pure LPS from *E. coli* strains are able to induce platelet activation, while others could not show this effect (Table 1B). Interestingly, LPSs are detected on platelet surfaces of children with EHEC-associated HUS, even before HUS developed, but not from children with uncomplicated EHEC infection [39].

Since there are also *E. coli* that secrete the shiga toxin (STEC), it is a well-studied bacterial product in the stimulation of platelet phenomena. Shiga toxin (Stx) is one of the most potent bacterial toxins known. The microbiologist Kiyoshi Shiga was the first to identify the bacterial origin of dysentery caused by *Shigella dysenteriae* in 1897. In 1977, a group of *E. coli* isolates was discovered by Konowalchuk that produce a factor capable of killing vero cells in culture, which is why they were named verotoxin-producing *E. coli* (VTEC) [40]. Shiga toxins (Stx), and verotoxins (VT), are a family of structurally and functionally related cytotoxic proteins produced by enteric pathogens such as Shigella dysenteriae type 1 and STEC.

The Shiga toxin family, a group of structurally and functionally related exotoxins, includes the Shiga toxin of *S. dysenteriae* serotype 1 and the Shiga toxins produced by EHEC. The Shiga toxin 1 produced by *E. coli* (Stx1) is almost identical to the family of Shiga toxin, differing by a single amino acid in the catalytic A subunit of the toxin. STEC (Shiga toxin-producing *E. coli)* can produce Stx1 variants (Stx1 and Stx1c), Stx2 variants (Stx2, Stx2c, Stx2d, Stx2e, Stx2f) or variants of both in a range of combinations [41]. Stx1 and Stx2 are genetically and even immunologically distinct and they share 55–60% genetic and amino acid identity, which is one 32 kDa A subunit and five identical 7.7 kDa B subunits [42–44]. Stx1 and 2 differ also in their localization. Stx1 is located in the periplasmic space of the bacterial cell, while Stx2 is in the extracellular fraction [45]. Stx1 is very similar to the Shiga toxin found in *Shigella dysenteriae* type 1. However, it has been demonstrated that Stx2 is 400 times more toxic than Stx1 in a murine infection model [46]. On the other hand, Stx2 is known to be more involved in complications of HUS than Stx1 [47], probably due to the fact that its A1 subunit has a higher affinity for ribosomes and a higher catalyzing potency than A1 subunit of Stx1, which makes it more cytotoxic than Stx1 [48].

Thrombocytopenia is one of the major clinical manifestations of HUS and is associated with the presence of Shiga toxin. Platelets are consumed during the formation of microthrombi and circulating platelets are degranulated [49]. Several studies describe potential interactions of Stx with platelets. Culture filtrates from Stx1-producing *E. coli* can cause platelet aggregation [50]. In parallel, Stx can bind directly to platelets. The toxin is apparently internalized in platelets after incubation, which leads to platelet aggregation. Morphological changes in platelets have been described that may contribute to the prothrombotic state seen in HUS [51], with Shiga toxin bound to activated platelets rather than to resting platelets [52].

3.3. Platelets—E. coli Interaction Consequences

The mechanism of platelet activation by Gram-negative bacteria, particularly *E. coli*, has been less studied than Gram-positive bacteria. Recent studies have shown that platelet activation and aggregation by *E. coli* are mainly dependent on the strain and the different receptors involved [53–56].

To our knowledge, only four studies have investigated the effect of *E. coli* strains on platelets (Table 1A) [53–56]. Among these studies, only Watson's work used non-EHEC clinical strains. They have demonstrated that platelet activation by the neuropathogenic and uropathogenic *E. coli* strains, CFT073 and RS218, is dependent on FcγRIIA via strain-bound plasma IgG [56]. In contrast, Fejes et al. using laboratory strains (K12 and 018:K1) obtained a different result depending on the strain. Indeed, only the non-pathogenic strain K12 induced platelet activation and granular secretion by a mechanism dependent on platelet glycoprotein IIb IIIa. Two hypotheses would explain this result. The expression of rough LPS, by strain K12, which would induce a more important cellular activation than the smooth LPS of strain O18 and the resistance to the complement of strain O18 could explain this difference of behaviour [53].

Works on EHEC strains have shown that the strains tested induce platelet activation and/or aggregation [54,55]. However, the mechanisms involved differ between the stages of haemostasis studied. Indeed, enterohemorrhagic the *E. coli* O111 strain induces platelet activation as evidenced by the expression of P-selectin and tissue factor. This process is TLR-4 dependent but independent of FcγRIIA [55]. In addition, Moriaty et al. were interested in platelet aggregation induced by the *E. coli* O157 strain. They demonstrated that platelet aggregation observed was TLR4- and FcγRIIA-dependent [55]. It could therefore be hypothesized that TLR4 is involved in activation phenomena. On the other hand, FcγRIIA would be necessary for platelet aggregation mechanisms.

The other studies looked specifically at the effect of pure LPS (Table 1B). The response of platelets to different LPS serotypes is not homogeneous; sometimes it is contradictory between studies, making the role of LPS in platelet activation and aggregation complex and dependent on several factors. LPS from *E. coli* O157 and other EHEC strains induced platelet binding and activation through the expression of GPIIb/IIIa, CD40L and fibrinogen binding. Activation was TLR4-dependent [39]. LPS from O111:B4, O55:B5 and O127:B8 caused platelet activation requiring TLR4/MD2, CD14 and MyD88 and induced platelet aggregation [31]. However, in another study, no platelet activation or aggregation was found with the same strain of *E. coli* O111:B4 LPS [57].

Ståhl et al. compared the effect of four pure LPSs of different serotypes (O157:H7, O103:H2, O111:HN, O121:H19) on platelet activation. Among the LPS tested, only O157:H7 LPS was able to induce higher platelet activation, which could be explained by the fact that there are differences in the LPS O-chain. LPS binding was abrogated by anti-TLR4 and anti-CD62P antibodies, suggesting that these receptors act in concert to bind LPS. However, a steric interaction between the antibodies and their receptors could not be excluded, due to the proximity of TLR4 and CD62 to the platelet surface [39].

In an interesting study, Moriarty et al. compared the effects of *E. coli* O157:H7 and LPS serotype from this strain. Only the whole bacterium induced platelet aggregation; no effect is observed with pure LPS. They suggested that the aggregation was not mediated by a direct interaction, but was enhanced by the action of the complement system [55].

Table 1. Summary of *E. coli* strains and *E. coli* pure LPS effect on platelets.

LPS/*E. coli* Strain	Strain Origin	Platelet Form	Reported Interaction Result	Platelet Receptor Involved	Reference
(A) *E. coli* Strain Effect on Platelet Activation and Aggregation					
CFT073 RS218	Pyelonephritis and bacteremia Neonatal meningitis	PRP	Activation and aggregation	FcγRIIA and αIIbβ3	[56]
K12	Non pathogen	PRP	Activation and dense granule release	GPIIb/IIIa	[53]
O18:K1	Pathogen	PRP	-	-	[53]
O111	EHEC	PRP	Activation	TLR4	[54]
O157:H7	EHEC	PRP	Aggregation	αIIbβ3/ Fc	[55]
(B) *E. coli* LPS Effect on Platelet Activation and Aggregation					
O111:B4	EHEC	PRP	-	-	[57]
O111:B4, O55:B5 and O127:B8	EHEC	PRP	Activation Alpha and dense granule secretion	CD14, TLR4/MD2 and MyD88	[31]
O157:H7	EHEC	PRP, WP	No aggregation	-	[55]
O103, O111, O121, O157O111:B4	EHEC Non-EHEC	PRP, WP	Activation and fibrinogen binding	GPIIb/III	[39]
LPS	Not mentioned	PRP WP	-	-	[58]

(-): No reported interaction consequence, PRP: Plasma Rich Platelets, WP: Washed Platelets. FcγRIIA: Fragment crystallizable gamma Receptor, αIIbβ3 or GPIIb/IIIa: integrin alpha IIb-beta-3, EHEC: Enterohemorrhagic *E. coli*, TLR-4: Toll-Like Receptors-4, MD2: myeloid differentiation 2, MyD88: Myeloid differentiation primary response 88, CD14: Cluster of Differentiation 14.

Concerning the aggregation phenomenon, studies suggest that the complement may stimulate platelet aggregation [59,60]. Other plasma proteins might enhance platelet activation and aggregation, such as PF4 [36,61,62]. As for platelet activation, the type of LPS could be another factor of variability of platelet aggregation.

Considering all these results, the platelet-*E. coli* interaction can be described as a complex one. The results of the mentioned studies cannot determine the factor(s) creating this heterogeneity of responses, but they suggest mechanisms which could help us to understand this interaction. First, most of the *E. coli* strains tested are those involved in HUS, which does not allow for a wide range of results for interpreting the difference in behavior of bacteria towards platelets and vice versa. In addition, Ståhl noted that the STEC strain has acquired virulence genes and islands associated with pathogenicity, which makes the development of HUS multifactorial [39]. Within these studies there are also differences among the protocols used. They differ in the preparation of platelets, bacterial concentration, platelet concentration, platelet–bacteria ratio and incubation time, especially as it is known that there is an interindividual variability of platelets that requires a normalization step for the number of platelets.

Platelets are also known for their inhibitory effect against microorganisms [4,63]. They can be included in the defense by chemotaxis by appealing to immune cells or directly by secreting antimicrobial molecules, called PMPs (platelet microbicidal peptides). Among these molecules, the most described are the following: platelet-derived chemokines, also known as kinocidins (e.g., CXCL4, CXCL7 (also known as PBP) and CCL5); defensins (e.g., human β-defensin 2 (BD2)); thymosin β4 (Tβ4); and derivative antimicrobial peptides (e.g., fibrinopeptide A or fibrinopeptide B and thrombocidins (which are proteolytic derivatives of CXCL7)) [64–66].

While studies have shown that platelets have a bactericidal effect against *Staphylococcus aureus* due to the antimicrobial molecules secreted as a result of platelet activation [67], this inhibitory effect on Gram-negative bacteria, in particular *E. coli*, is poorly described. The few available data (Table 2) show that the bactericidal effect on *E. coli* could depend on many factors, such as the strain tested and the platelet–bacteria ratio [54,68]. Indeed, the lack of consensus regarding a standard plasma rich platelet (PRP) preparation impairs any comparisons of the effectiveness of the inhibitory effect obtained by different research groups. Another issue is the inclusion or not of leukocytes, as PRP with leukocytes (L-PRP) presents different biologic activity, which could modify inhibitory activity [69].

Table 2. Summary of platelet inhibitory effect on *E. coli* strains.

E. coli Strain	Strain Origin	Platelet Form	Growth Inhibition Result	Factor Involved	Reference
ATCC 11303	Laboratory	PRP	+	hBD-2	[70]
ATCC 35218 ATCC 25922	Laboratory	L-PRP	–	-	[69]
ML35	Laboratory	Purified peptides from WP	+	pH = 5.5 Peptides concentration	[71]
K-12 WT	Laboratory	WP	–	-	[62]
KPM53 KPM121	Laboratory	WP	+	FcγRIIA and chemokine PF4	[62]

(+): Bacterial growth inhibition reported, (-): No growth inhibition reported, PRP: Plasma Rich Platelets, hBD-2: human Beta Defensin-2, L-PRP: leukocytes and plasma rich platelets, WP: Washed Platelets, FcγRIIA: Fragment crystallizable gamma Receptor, PF4: platelet factor 4.

Tang et al. identified seven human platelet antimicrobial proteins (HPAPs): fibrinopeptide A and B, thymosin beta 4, platelet basic protein, connective tissue-activating protein 3, RANTES and PF 4, which have antibacterial properties acting against *E. coli* ML-35 in a pH and concentration-dependent manner [71]. *E. coli* ATCC11303 was also mentioned to be inhibited by PRP, while re-incubation of PRP with anti-hBD-2 antibody (human beta defensin) revealed a significant decrease of antimicrobial activity [70].

Palankar et al. showed that platelets had direct FcγRIIA-mediated antimicrobial effects and also bridged innate and adaptive immunity via chemokine PF4. Platelets were able to kill two *E. coli* K12-derived (LPS mutant) strains in a PF4-dependent manner. However, this effect was less effective against the K12 wild-type strain. The bactericidal effect of platelets would be dependent on the structure of LPS [62]. Platelets appear to be able to discriminate between different forms of LPS. It has been shown that in vitro stimulation of platelets with LPS from *E. coli* O111 or *S. minnesota*, which are, respectively, smooth and rough types of LPS, differentially induces the release of immunomodulatory molecules and therefore a differential secretion of IL-6, TNF-α and IL-8 by peripheral blood mononuclear cells [72]. Different preparations of L-PRP tested on *E. coli* ESBL-ATCC 35218 and ATCC 25922 show no inhibitory activity on the growth of these two strains [69]. This result is in agreement with the work of Ketter et al., who showed that platelets do not inhibit the growth of *E. coli* ATCC 25922 but conversely promote it [73] (Table 2). So, the stimulation of platelets leading to the secretion of antibacterial molecules is also highly variable and dependent on several factors at the bacterial and platelet level.

4. Discussion

After analyzing studies on platelets and *E. coli*, we noticed that this is a two-way interaction, and it is highly variable (Figure 2). The consequences of interactions between platelets and different strains of *E. coli* induce highly variable effects on platelet functions. Several hypotheses could explain these differences in platelet behavior depending on the *E. coli* strains tested. The most highlighted hypothesis is the difference in the LPS form, more specifically the structure of O-antigen. This variability is due to sugar variation present in the O-unit and the linkages within and between O-units. This structural heterogeneity could explain the strain-specific differences in interaction with platelets. The loss of the O-antigen is associated with a decrease in virulence of the strains [74]. The O-specific polysaccharide is able to interact directly with lysozyme and is able to inhibit its hydrolytic activity. The O-specific polysaccharide is therefore able to protect bacteria against exogenous lysozyme [75].

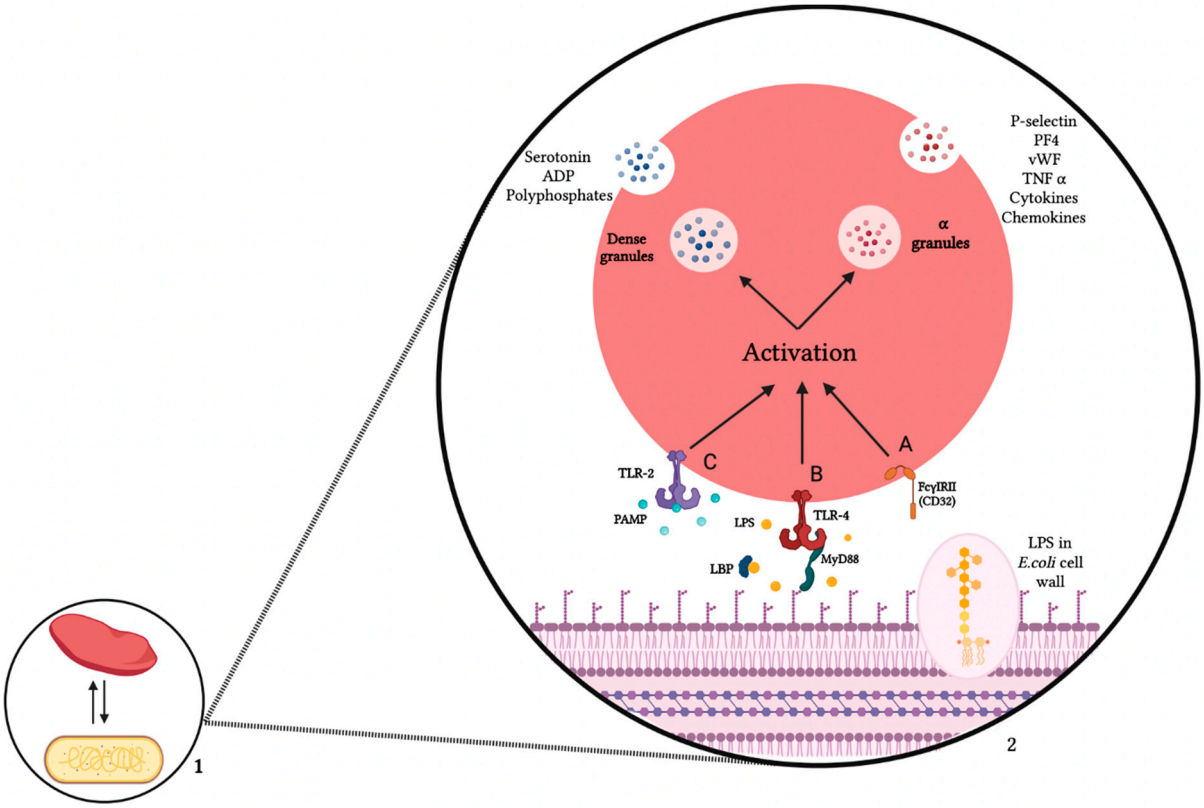

Figure 2. Interaction mechanisms between *E. coli* and platelets. (1) Two-way interaction between platelets and *E. coli*. (2) Mechanisms of interaction, (A) interaction via the FcγRIIA; (B) interaction via the binding between LPS and TLR4 with the intervention of MyD88 and LBP; (C) interaction with the involvement of the TLR2/PAMP complex. These three interaction mechanisms lead to platelet activation and subsequent release of alpha and dense granule contents. Created with BioRender.com, accessed on 15 February 2022. ADP: Adenosine diphosphate, PF4: Platelet Factor 4, vWF: von Willebrand factor, TNF: *Tumor Necrosis Factor*, TLR: Toll-Like Receptor, PAMP: pathogen-associated molecular pattern, LPS: Lipopolysaccharide, LBP: Lipopolysaccharide Binding Protein, MyD88: Myeloid differentiation primary response 88, FcγRIIA: Fragment Crystallizable gamma Receptor, *E. coli: Escherichia coli*.

Resistance mechanisms to antibiotics, such as polymyxin E (colistin), may also be a factor in these differences in the behavior of different *E. coli* strains towards platelets. Antibacterial platelet peptides, mainly called cationic antimicrobial peptides (CAMPs), such as defensins, show similarities in structure and polarity with colistin [76,77]. The most common mechanism of colistin resistance is due to chromosomal mutation of genes or acquisition of plasmid genes responsible for a modification of lipid A of LPS [78–80] which is the main target of colistin and platelets. It can therefore be hypothesized that colistin-resistant strains would also exhibit resistance to CAMP, possibly through a modification of lipid A. Resistance to CAMPs has been demonstrated in *Haemophilus ducreyi* and *Campylobacter jejuni*, two Gram-negative bacteria producing a phosphoethanolamine transferase that is the source of polymyxin resistance [76,81].

Involved in important pathologies, *E. coli* and its effect on platelets must be further studied by testing several strains in order to understand at the molecular level the mechanisms of activation, aggregation and release of antimicrobial molecules, especially since there are contradictory results even in studies on the same strain. The involvement of multiple platelet receptors must also be highlighted in order to be able to target and inhibit the factors leading to the development of *E. coli* pathologies. Given the significant role of FcγRIIa in platelet aggregation induced by Gram-positive and Gram-negative bacteria, FcγRIIa could be an interesting drug target for conditions such as sepsis.

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References

1. Thomas, M.R.; Storey, R.F. The Role of Platelets in Inflammation. *Thromb. Haemost.* **2015**, *114*, 449–458. [CrossRef] [PubMed]
2. Hannachi, N.; Fournier, P.-E.; Martel, H.; Habib, G.; Camoin-Jau, L. Statins Potentiate the Antibacterial Effect of Platelets on *Staphylococcus Aureus*. *Platelets* **2021**, *32*, 671–676. [CrossRef] [PubMed]
3. Yeaman, M.R. Bacterial-Platelet Interactions: Virulence Meets Host Defense. *Future Microbiol.* **2010**, *5*, 471–506. [CrossRef] [PubMed]
4. Yeaman, M.R. Platelets: At the Nexus of Antimicrobial Defence. *Nat. Rev. Microbiol.* **2014**, *12*, 426–437. [CrossRef]
5. Cox, D.; Kerrigan, S.W.; Watson, S.P. Platelets and the Innate Immune System: Mechanisms of Bacterial-Induced Platelet Activation. *J. Thromb. Haemost.* **2011**, *9*, 1097–1107. [CrossRef]
6. Hamzeh-Cognasse, H.; Damien, P.; Chabert, A.; Pozzetto, B.; Cognasse, F.; Garraud, O. Platelets and Infections-Complex Interactions with Bacteria. *Front. Immunol.* **2015**, *6*, 82. [CrossRef]
7. O'Seaghdha, M.; van Schooten, C.J.; Kerrigan, S.W.; Emsley, J.; Silverman, G.J.; Cox, D.; Lenting, P.J.; Foster, T.J. Staphylococcus Aureus Protein A Binding to von Willebrand Factor A1 Domain Is Mediated by Conserved IgG Binding Regions. *FEBS J.* **2006**, *273*, 4831–4841. [CrossRef]
8. Sarowska, J.; Futoma-Koloch, B.; Jama-Kmiecik, A.; Frej-Madrzak, M.; Ksiazczyk, M.; Bugla-Ploskonska, G.; Choroszy-Krol, I. Virulence Factors, Prevalence and Potential Transmission of Extraintestinal Pathogenic Escherichia Coli Isolated from Different Sources: Recent Reports. *Gut Pathog.* **2019**, *11*, 10. [CrossRef]
9. Kaper, J.B.; Nataro, J.P.; Mobley, H.L.T. Pathogenic Escherichia Coli. *Nat. Rev. Microbiol.* **2004**, *2*, 123–140. [CrossRef]
10. Leimbach, A.; Hacker, J.; Dobrindt, U.E. Coli as an All-Rounder: The Thin Line between Commensalism and Pathogenicity. *Curr. Top. Microbiol. Immunol.* **2013**, *358*, 3–32. [CrossRef]
11. Johnson, J.R.; Russo, T.A. Extraintestinal Pathogenic Escherichia Coli: "The Other Bad *E. Coli*". *J. Lab. Clin. Med.* **2002**, *139*, 155–162. [CrossRef] [PubMed]
12. Köhler, C.-D.; Dobrindt, U. What Defines Extraintestinal Pathogenic Escherichia Coli? *Int. J. Med. Microbiol.* **2011**, *301*, 642–647. [CrossRef] [PubMed]
13. Vila, J.; Sáez-López, E.; Johnson, J.R.; Römling, U.; Dobrindt, U.; Cantón, R.; Giske, C.G.; Naas, T.; Carattoli, A.; Martínez-Medina, M.; et al. Escherichia Coli: An Old Friend with New Tidings. *FEMS Microbiol. Rev.* **2016**, *40*, 437–463. [CrossRef] [PubMed]
14. Fitzhenry, R.J. Intimin Type Influences the Site of Human Intestinal Mucosal Colonisation by Enterohaemorrhagic Escherichia Coli O157:H7. *Gut* **2002**, *50*, 180–185. [CrossRef]
15. Yu, J.; Kaper, J.B. Cloning and Characterization of the Eae Gene of Enterohaemorrhagic Escherichia Coli O157:H7. *Mol. Microbiol.* **1992**, *6*, 411–417. [CrossRef]
16. Hernandes, R.T.; Elias, W.P.; Vieira, M.A.M.; Gomes, T.A.T. An Overview of Atypical Enteropathogenic Escherichia Coli. *FEMS Microbiol. Lett.* **2009**, *297*, 137–149. [CrossRef]
17. Jafari, A.; Aslani, M.M.; Bouzari, S. Escherichia Coli: A Brief Review of Diarrheagenic Pathotypes and Their Role in Diarrheal Diseases in Iran. *Iran J. Microbiol.* **2012**, *4*, 102–117.
18. Gouali, M.; Weill, F.-X. Enterohemorragic Escherichia coli (EHEC): Topical enterobacteriaceae. *Presse Med.* **2013**, *42*, 68–75. [CrossRef]
19. Botos, I.; Segal, D.M.; Davies, D.R. The Structural Biology of Toll-like Receptors. *Structure* **2011**, *19*, 447–459. [CrossRef]
20. Sameer, A.S.; Nissar, S. Toll-like Receptors (TLRs): Structure, Functions, Signaling, and Role of Their Polymorphisms in Colorectal Cancer Susceptibility. *BioMed Res. Int.* **2021**, *2021*, 1157023. [CrossRef]
21. Hally, K.; Fauteux-Daniel, S.; Hamzeh-Cognasse, H.; Larsen, P.; Cognasse, F. Revisiting Platelets and Toll-like Receptors (TLRs): At the Interface of Vascular Immunity and Thrombosis. *Int. J. Mol. Sci.* **2020**, *21*, E6150. [CrossRef] [PubMed]
22. Panigrahi, S.; Ma, Y.; Hong, L.; Gao, D.; West, X.Z.; Salomon, R.G.; Byzova, T.V.; Podrez, E.A. Engagement of Platelet Toll-like Receptor 9 by Novel Endogenous Ligands Promotes Platelet Hyperreactivity and Thrombosis. *Circ. Res.* **2013**, *112*, 103–112. [CrossRef] [PubMed]
23. Shiraki, R.; Inoue, N.; Kawasaki, S.; Takei, A.; Kadotani, M.; Ohnishi, Y.; Ejiri, J.; Kobayashi, S.; Hirata, K.; Kawashima, S.; et al. Expression of Toll-like Receptors on Human Platelets. *Thromb. Res.* **2004**, *113*, 379–385. [CrossRef] [PubMed]
24. de Stoppelaar, S.F.; Claushuis, T.A.M.; Schaap, M.C.L.; Hou, B.; van der Poll, T.; Nieuwland, R.; van 't Veer, C. Toll-like Receptor Signalling Is Not Involved in Platelet Response to Streptococcus Pneumoniae In Vitro or In Vivo. *PLoS ONE* **2016**, *11*, e0156977. [CrossRef]
25. Keane, C.; Tilley, D.; Cunningham, A.; Smolenski, A.; Kadioglu, A.; Cox, D.; Jenkinson, H.F.; Kerrigan, S.W. Invasive Streptococcus Pneumoniae Trigger Platelet Activation via Toll-like Receptor 2. *J. Thromb. Haemost.* **2010**, *8*, 2757–2765. [CrossRef] [PubMed]
26. Anabel, A.-S.; Eduardo, P.-C.; Pedro Antonio, H.-C.; Carlos, S.-M.; Juana, N.-M.; Honorio, T.-A.; Nicolás, V.-S.; Sergio Roberto, A.-R. Human Platelets Express Toll-like Receptor 3 and Respond to Poly I:C. *Hum. Immunol.* **2014**, *75*, 1244–1251. [CrossRef]
27. D' Atri, L.P.; Schattner, M. Platelet Toll-like Receptors in Thromboinflammation. *Front. Biosci.* **2017**, *22*, 1867–1883. [CrossRef]

28. Koupenova, M.; Mick, E.; Mikhalev, E.; Benjamin, E.J.; Tanriverdi, K.; Freedman, J.E. Sex Differences in Platelet Toll-like Receptors and Their Association With Cardiovascular Risk Factors. *Arterioscler. Thromb. Vasc. Biol.* **2015**, *35*, 1030–1037. [CrossRef]
29. Andonegui, G.; Kerfoot, S.M.; McNagny, K.; Ebbert, K.V.J.; Patel, K.D.; Kubes, P. Platelets Express Functional Toll-like Receptor-4. *Blood* **2005**, *106*, 2417–2423. [CrossRef]
30. Niklaus, M.; Klingler, P.; Weber, K.; Koessler, A.; Boeck, M.; Kobsar, A.; Koessler, J. The Involvement of Toll-like Receptors 2 and 4 in Human Platelet Signalling Pathways. *Cell. Signal.* **2020**, *76*, 109817. [CrossRef]
31. Zhang, G.; Han, J.; Welch, E.J.; Ye, R.D.; Voyno-Yasenetskaya, T.A.; Malik, A.B.; Du, X.; Li, Z. Lipopolysaccharide Stimulates Platelet Secretion and Potentiates Platelet Aggregation via TLR4/MyD88 and the CGMP-Dependent Protein Kinase Pathway. *J. Immunol.* **2009**, *182*, 7997–8004. [CrossRef] [PubMed]
32. Stark, R.J.; Aghakasiri, N.; Rumbaut, R.E. Platelet-Derived Toll-like Receptor 4 (Tlr-4) Is sufficient to promote microvascular thrombosis in endotoxemia. *PLoS ONE* **2012**, *7*, e41254. [CrossRef] [PubMed]
33. Stegner, D.; Nieswandt, B. Platelet Receptor Signaling in Thrombus Formation. *J. Mol. Med.* **2011**, *89*, 109–121. [CrossRef] [PubMed]
34. Qiao, J.; Al-Tamimi, M.; Baker, R.I.; Andrews, R.K.; Gardiner, E.E. The Platelet Fc Receptor, FcγRIIa. *Immunol. Rev.* **2015**, *268*, 241–252. [CrossRef]
35. Karas, S.P.; Rosse, W.F.; Kurlander, R.J. Characterization of the IgG-Fc Receptor on Human Platelets. *Blood* **1982**, *60*, 1277–1282. [CrossRef]
36. Arman, M.; Krauel, K.; Tilley, D.O.; Weber, C.; Cox, D.; Greinacher, A.; Kerrigan, S.W.; Watson, S.P. Amplification of Bacteria-Induced Platelet Activation Is Triggered by FcγRIIA, Integrin AIIbβ3, and Platelet Factor 4. *Blood* **2014**, *123*, 3166–3174. [CrossRef]
37. Raetz, C.R.H.; Whitfield, C. Lipopolysaccharide Endotoxins. *Annu. Rev. Biochem.* **2002**, *71*, 635–700. [CrossRef]
38. Lu, Y.-C.; Yeh, W.-C.; Ohashi, P.S. LPS/TLR4 Signal Transduction Pathway. *Cytokine* **2008**, *42*, 145–151. [CrossRef]
39. Ståhl, A.; Svensson, M.; Mörgelin, M.; Svanborg, C.; Tarr, P.I.; Mooney, J.C.; Watkins, S.L.; Johnson, R.; Karpman, D. Lipopolysaccharide from Enterohemorrhagic Escherichia Coli Binds to Platelets through TLR4 and CD62 and Is Detected on Circulating Platelets in Patients with Hemolytic Uremic Syndrome. *Blood* **2006**, *108*, 167–176. [CrossRef]
40. Konowalchuk, J.; Speirs, J.I.; Stavric, S. Vero Response to a Cytotoxin of Escherichia Coli. *Infect. Immun.* **1977**, *18*, 775–779. [CrossRef]
41. Eklund, M.; Leino, K.; Siitonen, A. Clinical Escherichia Coli Strains Carrying Stx Genes: Stx Variants and Stx-Positive Virulence Profiles. *J. Clin. Microbiol.* **2002**, *40*, 4585–4593. [CrossRef] [PubMed]
42. Strockbine, N.A.; Marques, L.R.; Newland, J.W.; Smith, H.W.; Holmes, R.K.; O'Brien, A.D. Two Toxin-Converting Phages from Escherichia Coli O157:H7 Strain 933 Encode Antigenically Distinct Toxins with Similar Biologic Activities. *Infect. Immun.* **1986**, *53*, 135–140. [CrossRef] [PubMed]
43. Weinstein, D.L.; Jackson, M.P.; Samuel, J.E.; Holmes, R.K.; O'Brien, A.D. Cloning and Sequencing of a Shiga-like Toxin Type II Variant from Escherichia Coli Strain Responsible for Edema Disease of Swine. *J. Bacteriol.* **1988**, *170*, 4223–4230. [CrossRef] [PubMed]
44. Fraser, M.E.; Chernaia, M.M.; Kozlov, Y.V.; James, M.N.G. Crystal Structure of the Holotoxino from Shigella Dysenteriae at 2.5 Å Resolution. *Nat. Struct. Mol. Biol.* **1994**, *1*, 59–64. [CrossRef]
45. Shimizu, T.; Ohta, Y.; Noda, M. Shiga Toxin 2 Is Specifically Released from Bacterial Cells by Two Different Mechanisms. *Infect. Immun.* **2009**, *77*, 2813–2823. [CrossRef]
46. Tesh, V.L.; Burris, J.A.; Owens, J.W.; Gordon, V.M.; Wadolkowski, E.A.; O'Brien, A.D.; Samuel, J.E. Comparison of the Relative Toxicities of Shiga-like Toxins Type I and Type II for Mice. *Infect. Immun.* **1993**, *61*, 3392–3402. [CrossRef]
47. Brooks, J.T.; Sowers, E.G.; Wells, J.G.; Greene, K.D.; Griffin, P.M.; Hoekstra, R.M.; Strockbine, N.A. Non-O157 Shiga Toxin–Producing *Escherichia Coli* Infections in the United States, 1983–2002. *J. Infect. Dis.* **2005**, *192*, 1422–1429. [CrossRef]
48. Basu, D.; Li, X.-P.; Kahn, J.N.; May, K.L.; Kahn, P.C.; Tumer, N.E. The A1 Subunit of Shiga Toxin 2 Has Higher Affinity for Ribosomes and Higher Catalytic Activity than the A1 Subunit of Shiga Toxin 1. *Infect. Immun.* **2016**, *84*, 149–161. [CrossRef]
49. Tarr, P.I. Shiga Toxin-Associated Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura: Distinct Mechanisms of Pathogenesis. *Kidney Int. Suppl.* **2009**, *75*, S29–S32. [CrossRef]
50. Rose, P.E.; Armour, J.A.; Williams, C.E.; Hill, F.G. Verotoxin and Neuraminidase Induced Platelet Aggregating Activity in Plasma: Their Possible Role in the Pathogenesis of the Haemolytic Uraemic Syndrome. *J. Clin. Pathol.* **1985**, *38*, 438–441. [CrossRef]
51. Karpman, D.; Papadopoulou, D.; Nilsson, K.; Sjögren, A.C.; Mikaelsson, C.; Lethagen, S. Platelet Activation by Shiga Toxin and Circulatory Factors as a Pathogenetic Mechanism in the Hemolytic Uremic Syndrome. *Blood* **2001**, *97*, 3100–3108. [CrossRef] [PubMed]
52. Ghosh, S.A.; Polanowska-Grabowska, R.K.; Fujii, J.; Obrig, T.; Gear, A.R.L. Shiga Toxin Binds to Activated Platelets. *J. Thromb. Haemost.* **2004**, *2*, 499–506. [CrossRef]
53. Fejes, A.V.; Best, M.G.; van der Heijden, W.A.; Vancura, A.; Verschueren, H.; de Mast, Q.; Wurdinger, T.; Mannhalter, C. Impact of Escherichia Coli K12 and O18:K1 on Human Platelets: Differential Effects on Platelet Activation, RNAs and Proteins. *Sci. Rep.* **2018**, *8*, 16145. [CrossRef] [PubMed]
54. Matus, V.; Valenzuela, J.G.; Hidalgo, P.; Pozo, L.M.; Panes, O.; Wozniak, A.; Mezzano, D.; Pereira, J.; Sáez, C.G. Human Platelet Interaction with E. Coli O111 Promotes Tissue-Factor-Dependent Procoagulant Activity, Involving Toll like Receptor 4. *PLoS ONE* **2017**, *12*, e0185431. [CrossRef] [PubMed]

55. Moriarty, R.D.; Cox, A.; McCall, M.; Smith, S.G.J.; Cox, D. *Escherichia Coli* Induces Platelet Aggregation in an FcγRIIa-Dependent Manner. *J. Thromb. Haemost.* **2016**, *14*, 797–806. [CrossRef] [PubMed]
56. Watson, C.N.; Kerrigan, S.W.; Cox, D.; Henderson, I.R.; Watson, S.P.; Arman, M. Human Platelet Activation by Escherichia Coli: Roles for FcγRIIA and Integrin AIIbβ3. *Platelets* **2016**, *27*, 535–540. [CrossRef] [PubMed]
57. Vallance, T.M.; Ravishankar, D.; Albadawi, D.A.I.; Layfield, H.; Sheard, J.; Vaiyapuri, R.; Dash, P.; Patel, K.; Widera, D.; Vaiyapuri, S. Effect of Ultrapure Lipopolysaccharides Derived from Diverse Bacterial Species on the Modulation of Platelet Activation. *Sci. Rep.* **2019**, *9*, 18258. [CrossRef]
58. Claushuis, T.A.M.; Van Der Veen, A.I.P.; Horn, J.; Schultz, M.J.; Houtkooper, R.H.; Van 'T Veer, C.; Van Der Poll, T. Platelet Toll-like Receptor Expression and Activation Induced by Lipopolysaccharide and Sepsis. *Platelets* **2019**, *30*, 296–304. [CrossRef]
59. Orth-Höller, D.; Würzner, R. Role of Complement in Enterohemorrhagic Escherichia Coli-Induced Hemolytic Uremic Syndrome. *Semin. Thromb. Hemost.* **2014**, *40*, 503–507. [CrossRef]
60. Würzner, R.; Riedl, M.; Rosales, A.; Orth-Höller, D. Treatment of Enterohemorrhagic Escherichia Coli-Induced Hemolytic Uremic Syndrome (EHUS). *Semin. Thromb. Hemost.* **2014**, *40*, 508–516. [CrossRef]
61. Krauel, K.; Pötschke, C.; Weber, C.; Kessler, W.; Fürll, B.; Ittermann, T.; Maier, S.; Hammerschmidt, S.; Bröker, B.M.; Greinacher, A. Platelet Factor 4 Binds to Bacteria, [Corrected] Inducing Antibodies Cross-Reacting with the Major Antigen in Heparin-Induced Thrombocytopenia. *Blood* **2011**, *117*, 1370–1378. [CrossRef] [PubMed]
62. Palankar, R.; Kohler, T.P.; Krauel, K.; Wesche, J.; Hammerschmidt, S.; Greinacher, A. Platelets Kill Bacteria by Bridging Innate and Adaptive Immunity via Platelet Factor 4 and FcγRIIA. *J. Thromb. Haemost.* **2018**, *16*, 1187–1197. [CrossRef] [PubMed]
63. Li, C.; Li, J.; Ni, H. Crosstalk Between Platelets and Microbial Pathogens. *Front. Immunol.* **2020**, *11*, 1962. [CrossRef] [PubMed]
64. Aquino-Domínguez, A.S.; Romero-Tlalolini, M.d.L.A.; Torres-Aguilar, H.; Aguilar-Ruiz, S.R. Recent Advances in the Discovery and Function of Antimicrobial Molecules in Platelets. *Int. J. Mol. Sci.* **2021**, *22*, 10230. [CrossRef]
65. Krijgsveld, J.; Zaat, S.A.; Meeldijk, J.; van Veelen, P.A.; Fang, G.; Poolman, B.; Brandt, E.; Ehlert, J.E.; Kuijpers, A.J.; Engbers, G.H.; et al. Thrombocidins, Microbicidal Proteins from Human Blood Platelets, Are C-Terminal Deletion Products of CXC Chemokines. *J. Biol. Chem.* **2000**, *275*, 20374–20381. [CrossRef]
66. Pasupuleti, M.; Schmidtchen, A.; Malmsten, M. Antimicrobial Peptides: Key Components of the Innate Immune System. *Crit. Re.v Biotechnol.* **2012**, *32*, 143–171. [CrossRef]
67. Hannachi, N.; Grac, L.; Baudoin, J.-P.; Fournier, P.-E.; Habib, G.; Camoin-Jau, L. Effect of Antiplatelet Agents on Platelet Antistaphylococcal Capacity: An in Vitro Study. *Int. J. Antimicrob. Agents* **2020**, *55*, 105890. [CrossRef]
68. Kerrigan, S.W.; Cox, D. Platelet-Bacterial Interactions. *Cell. Mol. Life Sci.* **2010**, *67*, 513–523. [CrossRef]
69. Cieślik-Bielecka, A.; Bold, T.; Ziółkowski, G.; Pierchała, M.; Królikowska, A.; Reichert, P. Antibacterial Activity of Leukocyte- and Platelet-Rich Plasma: An *In Vitro* Study. *BioMed Res. Int.* **2018**, *2018*, 9471723. [CrossRef]
70. Tohidnezhad, M.; Varoga, D.; Wruck, C.J.; Podschun, R.; Sachweh, B.H.; Bornemann, J.; Bovi, M.; Sönmez, T.T.; Slowik, A.; Houben, A.; et al. Platelets Display Potent Antimicrobial Activity and Release Human Beta-Defensin 2. *Platelets* **2012**, *23*, 217–223. [CrossRef]
71. Tang, Y.-Q.; Yeaman, M.R.; Selsted, M.E. Antimicrobial Peptides from Human Platelets. *Infect. Immun.* **2002**, *70*, 6524–6533. [CrossRef] [PubMed]
72. Kappelmayer, J.; Beke Debreceni, I.; Vida, A.; Antal-Szalmás, P.; Clemetson, K.J.; Nagy, B. Distinct Effects of Re- and S-Forms of LPS on Modulating Platelet Activation. *J. Thromb. Haemost.* **2013**, *11*, 775–778. [CrossRef] [PubMed]
73. Ketter, P.M.; Kamucheka, R.; Arulanandam, B.; Akers, K.; Cap, A.P. Platelet Enhancement of Bacterial Growth during Room Temperature Storage: Mitigation through Refrigeration. *Transfusion* **2019**, *59*, 1479–1489. [CrossRef] [PubMed]
74. Plainvert, C.; Bidet, P.; Peigne, C.; Barbe, V.; Médigue, C.; Denamur, E.; Bingen, E.; Bonacorsi, S. A New O-Antigen Gene Cluster Has a Key Role in the Virulence of the Escherichia Coli Meningitis Clone O45:K1:H7. *J. Bacteriol.* **2007**, *189*, 8528–8536. [CrossRef]
75. Bao, Y.; Zhang, H.; Huang, X.; Ma, J.; Logue, C.M.; Nolan, L.K.; Li, G. O-Specific Polysaccharide Confers Lysozyme Resistance to Extraintestinal Pathogenic Escherichia Coli. *Virulence* **2018**, *9*, 666–680. [CrossRef]
76. Andersson, D.I.; Hughes, D.; Kubicek-Sutherland, J.Z. Mechanisms and Consequences of Bacterial Resistance to Antimicrobial Peptides. *Drug Resis. Updat.* **2016**, *26*, 43–57. [CrossRef]
77. Hancock, R.E.; Diamond, G. The Role of Cationic Antimicrobial Peptides in Innate Host Defences. *Trends Microbiol.* **2000**, *8*, 402–410. [CrossRef]
78. Hamel, M.; Rolain, J.-M.; Baron, S.A. The History of Colistin Resistance Mechanisms in Bacteria: Progress and Challenges. *Microorganisms* **2021**, *9*, 442. [CrossRef]
79. Baron, S.; Hadjadj, L.; Rolain, J.-M.; Olaitan, A.O. Molecular Mechanisms of Polymyxin Resistance: Knowns and Unknowns. *Int. J. Antimicrob. Agents* **2016**, *48*, 583–591. [CrossRef]
80. Hadjadj, L.; Riziki, T.; Zhu, Y.; Li, J.; Diene, S.M.; Rolain, J.-M. Study of Mcr-1 Gene-Mediated Colistin Resistance in Enterobacteriaceae Isolated from Humans and Animals in Different Countries. *Genes* **2017**, *8*, E394. [CrossRef]
81. Napier, B.A.; Burd, E.M.; Satola, S.W.; Cagle, S.M.; Ray, S.M.; McGann, P.; Pohl, J.; Lesho, E.P.; Weiss, D.S. Clinical Use of Colistin Induces Cross-Resistance to Host Antimicrobials in Acinetobacter Baumannii. *mBio* **2013**, *4*, e00021-13. [CrossRef] [PubMed]

Chapitre II : Évaluation de l'effet antimicrobien des plaquettes sur la croissance d'*Escherichia coli*

Préambule

Dans ce chapitre, nous nous sommes intéressées à l'action bactéricide des plaquettes sur dix souches d'*E. coli*. En effet, les plaquettes peuvent être considérées comme des cellules de défense contre les agents pathogènes par chimiotaxie en faisant appel aux cellules immunitaires ou directement en sécrétant des molécules antimicrobiennes, appelées PMPs (peptides microbicides plaquettaires) (11,37). Ces molécules sont libérées à la suite d'une activation plaquettaire, induite par des agents pathogènes lors d'une infection. Selon leur localisation, deux types de molécules plaquettaires pourraient être distinguées, les molécules granulaires et extra-granulaires. Les chimiokines comme le facteur plaquettaire-4 (PF-4) et CCL5, les thrombocidines 1-2 et la thymosine β4 (Tβ4) sont stockées dans les granules alpha (8–10), tandis que les β-défensines humaines- 1, 2 et 3 (HβD-1,2 et 3) sont indépendantes du processus de dégranulation (35,38,39). Ces HβDs représentent une sous-famille importante des PMPs qui tuent les microbes en détruisant leurs membranes cellulaires sans l'utilisation du système immunitaire adaptatif (35,40). Elles ont également une charge cationique ce qui est semblable aux caractéristiques d'un antibiotique, la polymyxine E, également appelée « colistine » (41,42).

Bien que des études aient démontré que les plaquettes ont un effet bactéricide contre *Staphylococcus aureus* grâce aux molécules antimicrobiennes sécrétées à la suite de l'activation plaquettaire (16,19), cet effet inhibiteur sur les bactéries Gram-négatif, en particulier *E. coli* est mal décrit. Les quelques données de la littérature disponibles montrent que l'effet bactéricide sur *E. coli* est hautement variable, il pourrait dépendre de nombreux facteurs, tels que la souche testée et le ratio plaquettes-bactéries (30,43).

Ce travail comporte deux parties principales : la première a pour objectif d'évaluer l'effet antibactérien des plaquettes sur plusieurs souches d'*E. coli,* soit isolées à partir des échantillons cliniques soit des souches de laboratoire. Ces souches ont des profils sensibles ou résistantes à la colistine. La seconde partie a été consacrée à l'évaluation de l'effet de ces différentes souches sur l'activation plaquettaire.

Nos résultats ont démontré que la capacité antibactérienne des plaquettes est principalement souche-dépendante. En effet, après leur incubation avec les bactéries, les plaquettes ont inhibé la croissance de trois souches seulement, que nous avons nommé plaquettes-sensibles, sur dix souches testées. À l'opposé, la croissance des autres souches est augmentée après l'incubation avec les plaquettes. Ces souches sont dites plaquettes-résistantes. En parallèle, les résultats de cytométrie en flux indiquent que certaines souches augmentent la

production de la P-selectine, ce qui traduit une activation plaquettaire avec des niveaux variables. À l'inverse, d'autres souches n'ont aucun un effet significatif sur l'activation. Nos résultats ont démontré que l'activité bactéricide est liée au niveau d'activation. En effet, une corrélation entre le nombre de colonies en présence de plaquettes et le pourcentage d'activation a été démontrée par une analyse statistique. Afin d'étudier si la croissance de certaines souches en présence de plaquettes est la conséquence d'un défaut de contact ou à une résistance aux PMPs, nous avons utilisé le surnageant des plaquettes préalablement activées. Ce surnageant a été incubé avec les souches dont la croissance est favorisée par les plaquettes. Le surnageant n'a pas d'effet inhibiteur sur la croissance des souches dites plaquettes-résistantes. Ces résultats démontrent que le mécanisme d'inhibition est d'origine sécrétoire.

Afin de comprendre cette variabilité de réponse, nous avons réalisé une analyse génomique afin de rechercher une éventuelle similarité de séquence entre les souches qui ont le même profil vis-à-vis des plaquettes. Les relations phylogénétiques basées sur le pangénome ne montrent pas de classification corrélée au profils obtenus. Afin de comprendre nos résultats, nous avons étudié la structure du LPS. En effet, il a été démontré que la forme du LPS pourrait être un facteur de variabilité (33). Le LPS-R peut activer un nombre plus important de type cellulaire (45,46). Les plaquettes aussi peuvent discriminer différentes formes de LPS. Dans le cas de *Salmonella minnesota* le LPS rugueux active les plaquettes par le TLR-4 (47).

Enfin, une comparaison des séquences de synthèse de l'antigène-O entre deux souches apparentées mais ayant des profils différents, a montré qu'une modification génétique pourrait causer un défaut d'expression de l'antigène-O et donc un comportement différent vis-à-vis des plaquettes.

En conclusion, nos travaux ont évalué l'effet bactéricide des plaquettes sur dix souches d'*E. coli*. D'une part, nous avons mis en évidence une corrélation entre l'activation plaquettaire induite par *E. coli* et l'activité bactéricide des plaquettes. Toutes les souches cliniques testées dans notre étude ont montré une résistance aux peptides plaquettaires et leur croissance bactérienne est augmentée en présence de plaquettes. Il est important d'approfondir les mécanismes de cette interaction pour bien comprendre et identifier les facteurs impliqués.

Article 2: The Antibacterial Effect of Platelets on *Escherichia coli* Strains.

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Article

The Antibacterial Effect of Platelets on *Escherichia coli* Strains

Amina Ezzeroug Ezzraimi [1,2], Nadji Hannachi [1,3], Antoine Mariotti [1,2,4], Clara Rolland [2,5], Anthony Levasseur [2,5], Sophie Alexandra Baron [1,2], Jean-Marc Rolain [1,2] and Laurence Camoin-Jau [1,2,4,*]

1 Aix Marseille University, IRD, APHM, MEPHI, IHU Méditerranée Infection, 13385 Marseille, France; amina.ezzeroug.ezzraimi@gmail.com (A.E.E.); n_adji07@live.fr (N.H.); antoine-julien.mariotti@ap-hm.fr (A.M.); sophie.baron.2@univ-amu.fr (S.A.B.); jean-marc.rolain@univ-amu.fr (J.-M.R.)
2 IHU Méditerranée Infection, Boulevard Jean Moulin, 13385 Marseille, France; rolland.clara@sfr.fr (C.R.); anthony.levasseur@univ-amu.fr (A.L.)
3 Département de Pharmacie, Faculté de Médecine, Université Ferhat Abbas Sétif I, Sétif 19000, Algeria
4 Laboratoire d'Hématologie, Hôpital de la Timone, APHM, Boulevard Jean-Moulin, 13385 Marseille, France
5 Aix Marseille University, IRD, SSA, APHM, VITROME, IHU Méditerranée Infection, 13385 Marseille, France
* Correspondence: laurence.camoin@ap-hm.fr; Tel.: +33-4-13-73-24-01; Fax: +33-4-13-73-24-02

Abstract: Platelets play an important role in defense against pathogens; however, the interaction between *Escherichia coli* and platelets has not been well described and detailed. Our goal was to study the interaction between platelets and selected strains of *E. coli* in order to evaluate the antibacterial effect of platelets and to assess bacterial effects on platelet activation. Washed platelets and supernatants of pre-activated platelets were incubated with five clinical colistin-resistant and five laboratory colistin-sensitive strains of *E. coli* in order to study bacterial growth. Platelet activation was measured with flow cytometry by evaluating CD62P expression. To identify the difference in strain behavior toward platelets, a pangenome analysis using Roary and O-antigen serotyping was carried out. Both whole platelets and the supernatant of activated platelets inhibited growth of three laboratory colistin-sensitive strains. In contrast, platelets promoted growth of the other strains. There was a negative correlation between platelet activation and bacterial growth. The Roary results showed no logical clustering to explain the mechanism of platelet resistance. The diversity of the responses might be due to strains of different types of O-antigen. Our results show a bidirectional interaction between platelets and *E. coli* whose expression is dependent on the bacterial strain involved.

Keywords: *Escherichia coli*; platelets; O-antigen; lipopolysaccharide

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1. Introduction

Platelets have been widely described as the main actors in hemostasis and thrombosis. More recently, the important role they play in inflammation and defense against pathogens has been highlighted [1,2]. Bacteria interact with platelets through three main mechanisms: (i) by direct binding: bacteria can express surface proteins which allow them to interact directly with surface receptors on platelets and bind to them, as in the case of *Streptococcus sanguinis*, which showed the ability to bind directly to GPIbα [3]; (ii) by binding through plasma proteins: bacterial proteins are capable of binding to fibrinogen and vWF (von Willebrand Factor), serving as a bridge between two cells, as in the example of *Staphylococcus aureus* expressing surface protein A (SpA) that binds to vWf and which in turn binds to platelet GPIbα [4]; (iii) and by binding through the secretion of bacterial products, such as toxins, which interact with platelets and activate them. Among these molecules, the Shiga toxin secreted by *Escherichia coli* induces platelet aggregation by binding to glycosphingolipid receptors on the surface of platelets [5–10]. This difference in interaction mechanisms, which is mainly dependent on the bacteria, induces distinct platelet responses. In most cases, this interaction leads to platelet activation, followed by a release of their granular contents composed of, among other things, microbicidal

proteins and chemokines that facilitate the destruction of pathogens, signal immune cells and promote inflammation [11,12].

Platelet microbicidal effects have been extensively studied for Gram-positive bacteria. It has been demonstrated that platelets decrease the growth of *Staphylococcus aureus* [13–15]. In contrast, this effect has been less studied for Gram-negative bacteria, and such data are scarce. Platelets have been shown to interact with *Escherichia coli* through the platelet Toll-Like Receptor 4 (TLR4) and bacterial lipopolysaccharide (LPS) [5,16,17]. The initiation of the pro-inflammatory signal by LPS depends on the interaction between the TLR4 complex and lipid A, a fragment of LPS [18]. However, other research has shown that platelet activation and aggregation occurs through FcγRIIA without the involvement of TLR4 [19,20]. In contrast, Matus et al. demonstrated that platelet activation is dependent on TLR4 but without FcγRIIA engagement [21]. It is important to note that studies have been carried out to investigate the effects of *E. coli* on the activation and aggregation of platelets, specifically in the case of hemolytic uremic syndrome (HUS), but very few data are available on the effects of platelets on *E. coli*. These studies have tested distinct strains and serotypes and different operating protocols, such as the platelet-bacteria ratio and platelet form [21–23].

The aim of this study was to investigate the interaction between platelets and different human *E. coli* strains by initially evaluating the antibacterial effect of platelets, and then evaluating the effect of *E. coli* strains on platelet activation. Moreover, our objective was to compare ten strains of *E coli* having different characteristics, including their colistin sensitivity profile and their pathogenic capability.

2. Materials and Methods

2.1. Platelet Preparation

Blood was drawn by venepuncture in sodium citrate from healthy subjects who were not receiving antibiotics, anti-inflammatory, or anti-platelet drugs. Platelet rich plasma (PRP) was prepared according to International Society on Thrombosis and Hemostasis (ISTH) recommendations [24]. A platelet count was performed using a hematology analyzer. PRP was again centrifuged at 1100 g for ten minutes to obtain a platelet pellet that was suspended in phosphate buffered saline (PBS) to obtain a solution of 4×10^9/L. Platelets were then kept at 37 °C in order to prevent activation. The protocol was approved by the ethics committee of the IHU Méditérrannée Infection (Reference 2016–002). All of the subjects gave their written informed consent in accordance with the Declaration of Helsinki.

2.2. Bacterial Preparation

In order to test strains of *E. coli* against platelets and to see if there is a possible cross-resistance between resistance to colistin and resistance to platelet antimicrobial peptides, ten strains of *E. coli* were selected. Five laboratory colistin-sensitive strains were used (ATCC 25922, ATCC 11303, K12, J53 and BL21DE3). Five colistin-resistant human isolates stored at the IHU were also tested (LH1, LH30, Q1065, Q1066 and Q6269) (Table 1). Identification was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and the Biotyper database (Bruker, Dresden, Germany). Strains were grown at 37 °C in an overnight culture of Columbia agar +5% sheep blood (bioMérieux, Marcy l'Etoile, France). After 18 h of incubation at 37 °C, the colonies were removed and suspended in 0.9% NaCl medium to obtain the required concentrations: 1×10^8 CFU (Colony Forming Unit)/mL for flow cytometry and 3×10^8 CFU/mL for growth test.

For each strain, the minimum inhibitory concentration (MIC) of colistin (Table 1) was tested by microdilution in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Table 1. Origins and characteristics of *E. coli* strains used in this study.

Escherichia coli Strain	Origin	O-Antigen Type	Colistin Resistance Mechanism	MIC	References
IHU clinical isolates					
LH 1	Human	O174	*mcr-1* gene	7.8 mg/L	[25]
LH 30	Human	O8	*mcr-1* gene	3.9 mg/L	[25]
Q1066	Human	O25	Unknow mechanism	7.8 mg/L	Unpublished
Q1065	Human (Pharyngeal swab)	O9	Unknow mechanism	3.9 mg/L	Unpublished
Q6269	Human (urine)	O175	Unknow mechanism	3.9 mg/L	Unpublished
Laboratory strains					
ATCC 25922	Reference strain	O6	-	0.97 mg/L	[26]
ATCC 11303	Reference strain	O7	-	0.48 mg/L	[27]
K12	Human	-	-	1.95 mg/L	[28]
J53	Laboratory mutant of K12	O16	-	0.97 mg/L	[29]
BL 21 DE3	Laboratory mutant of K12	O7	-	0.97 mg/L	[30]

2.3. Analysis of Platelet Activation by Flow Cytometry

The washed platelets were used at a concentration of 2.5 × 10^9/L, adjusting the concentration with PBS. They were incubated with bacteria in a 1:2.5 bacteria-platelet ratio for one hour at 37 °C and identified by expression of the CD41-FITC antibody (4 µL, IgG, Beckman Coulter, Villepinte, France), as previously described [31]. To determine possible platelet activation following incubation with the bacteria, the expression of the CD62-PC5 antibody (4 µL, IgG, monoclonal, BD sciences, San Jose, CA, USA) on their surface was measured using flow cytometry (Beckman Coulter, FC500, Fullerton, CA, USA). The platelet activator, Thrombin receptor-activating peptide 6 (TRAP) (STAGO®, Asnières, France) (10 µM), and untreated platelets were used as controls. The results were represented by a Mean fluorescence intensity (MFI) percentage of P-Selectin; the expression was calculated using the following equation: (MFI of platelets incubated with bacteria) × 100/(MFI of platelets alone).

2.4. Platelet Supernatant Effect on Bacterial Growth

To obtain the supernatant from the activated platelets, the activated platelets were incubated with the J53 strain for one hour at 37 °C. The mixture underwent three successive centrifugation rounds (1300× *g*, 5000× *g* and 5000× *g*; ten minutes each) and the supernatant was recovered and filtered (0.22 µm), then incubated with bacteria for four hours at 37 °C. Mixtures were serially diluted as described above, then spread on agar and the colonies were counted the following day.

2.5. Pangenome Analysis

The genomes of the seven strains of *Escherichia coli* (ATCC25922, ATCC11303, K12, J53, BL21 DE3, LH1 and LH30) were retrieved from the National Centre for Biotechnology Information (NCBI) database. For the three other strains, Q1065 (unpublished data), Q1066 (unpublished data) and Q6269 (JAIBLN000000000), the genomes were obtained from the IHU sequencing platform by Illumina MiSeq according to a paired-end strategy.

The first step in performing the pangenome was to predict the ORFs (Open Reading Frame) for each strain with PROKKA software using the default parameters [32]. Then, Roary software was used to build the pangenome with the core genome alignment default

parameters [33]. A graphic representation of the pangenome results was prepared using the roary_plots.py script provided on the Roary website.

2.6. O-antigen Strain Serotyping

The serotyping of the *E. coli* strains (Table 1) used in this study was performed in silico from the genomes obtained by high-throughput sequencing. The FASTA sequences of these genomes were analyzed using ECTyper software [34], which makes it possible to serotype the O and H antigens of *E. coli* and *Shigella* spp. The bioinformatic predictions made with this software were then compared with those obtained by the Serotype Finder 2.0 prediction module of the Centre for Genomic Epidemiology developed by the Technical University of Denmark [35]. The results of all of the strains were concordant after analysis by both databases.

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 for Windows (GraphPad Software, La Jolla, CA, USA). Significant differences (for bacterial growth and flow cytometry) between the two groups were determined using the two-tailed, paired Student's *t*-test. The statistical significance was set at $p < 0.05$. A test of normality was applied on the effect of platelets on bacterial growth data, and it turned out that samples (negative control and platelets + bacteria for each strain) show a Gaussian distribution following a verification by the Shapiro-Wilk test ($p > 0.05$).

While for supernatants effect, they were determined using the Bonferroni's multiple comparisons test precede by Two-way ANOVA test, considering $p < 0.05$ as statistically significant. The correlation between bacterial growth and MFI percentage values was determined using the Pearson test.

3. Results

3.1. The Effect of Platelets on the Growth of E. coli Strains

After four hours of incubation, the platelets significantly decreased bacterial growth of three *E. coli* laboratory strains, as compared to the controls ($n = 5$ for each strain: $p = 0.0036$, <0.0001 and 0.0017 for ATCC11303, BL21DE3 and J53, respectively, paired Student's *t*-test) (Figure 1). These strains were referred to as "platelet sensitive".

In contrast, the platelets significantly promoted the growth of *E. coli* K12 and *E. coli* ATCC25922 ($n = 5$ for each strain: $p = 0.0013$ and 0.0065, respectively). Likewise, the growth of all the clinical strains was enhanced with platelets ($n = 5$ for each strain: $p = 0.0001$, 0.0005, 0.0105, 0.0009 and 0.0079, for LH1, LH30, Q1065, Q1066, and Q6269, respectively, paired Student's *t*-test) (Figure 1). These seven strains were referred to as "platelet resistant".

3.2. The Effect of Platelet Supernatant on the Growth of E. coli Strains

Mixes of the supernatants of platelets previously stimulated by the *E. coli* J53 strain were prepared, then re-incubated again with bacteria, as described in the Section 2. The supernatant of the platelets treated by TRAP was used as a positive control.

The supernatants showed effects similar to whole platelets on the growth of tested strains. Indeed, supernatants of platelets stimulated with *E. coli* J53 and TRAP significantly inhibited the growth of *E. coli* BL21DE3 and *E. coli* ATCC 11303 strains (($n = 5$: $p = 0.0223$, 0.0293 and $p = 0.0381$, 0.0048 for BL21 and J53, respectively, Figure 2A). In contrast, the supernatants of platelets stimulated with *E. coli* J53 and TRAP continued to promote the growth of K12 ($n = 5$: $p = 0.0203$ and 0.0332, Figure 2B). Q1065 growth was also enhanced by supernatants of platelets stimulated with *E. coli* J53 ($n = 5$: $p = 0.0233$). On the other hand, platelet supernatants had no significant effect on ATCC 25922 growth.

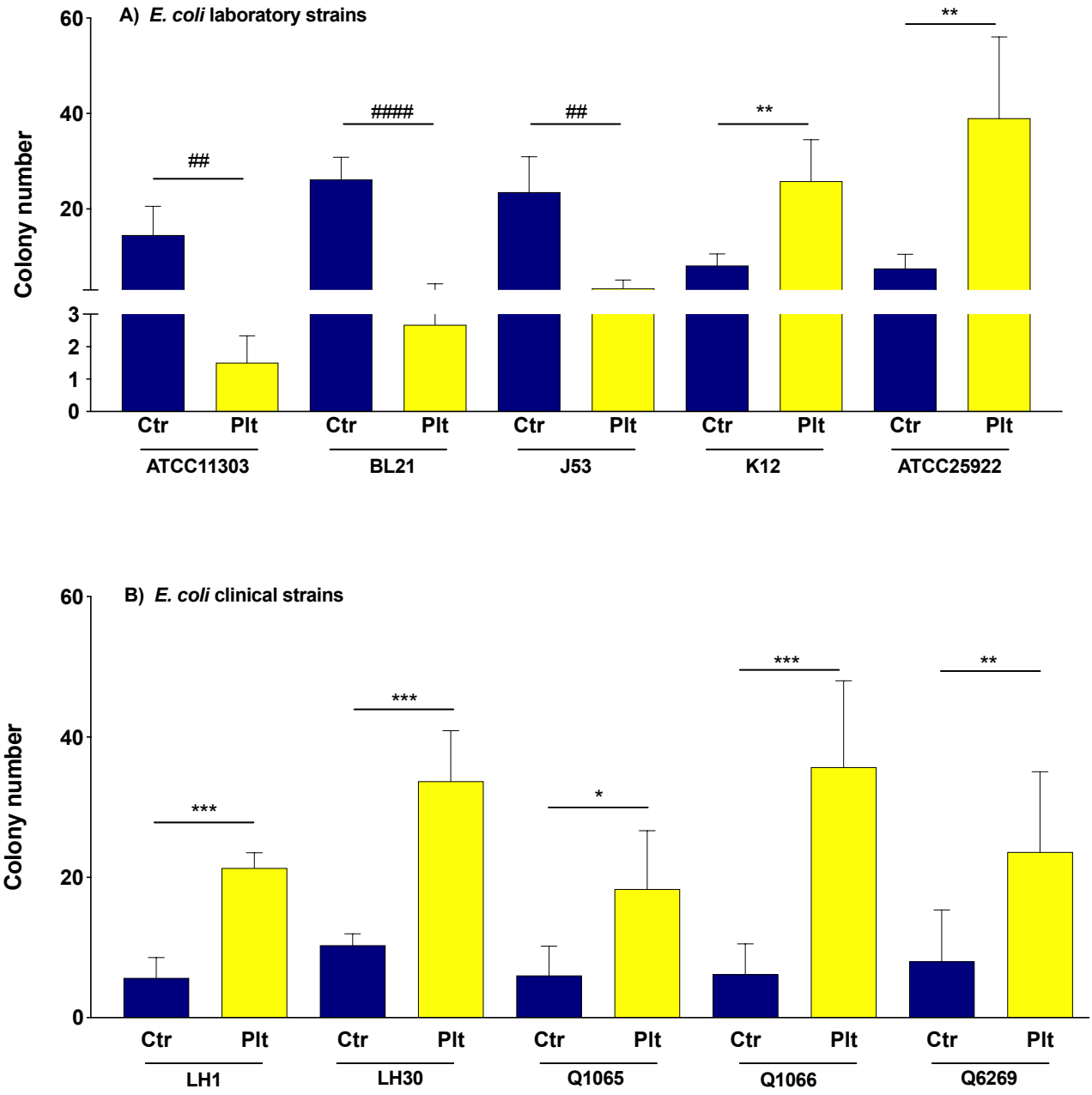

Figure 1. The effect of platelets on the growth of *E. coli* strains. Bacteria (20 μL, 3×10^8 CFU) were added to platelets (180 μL, 4×10^9/L) and incubated together at 37 °C for four hours while being rotated. Bars represent Mean with SD. Ctr = control: bacteria alone (blue column). Plt = bacteria-platelet mixture 1:10 ratio (yellow column). *: significant increase; #: significant decrease. Significant differences between the two groups were determined using the two-tailed, paired Student's *t*-test. *: $p < 0.05$, ** and ##: $p < 0.01$, ***: $p < 0.001$, ####: $p < 0.0001$.

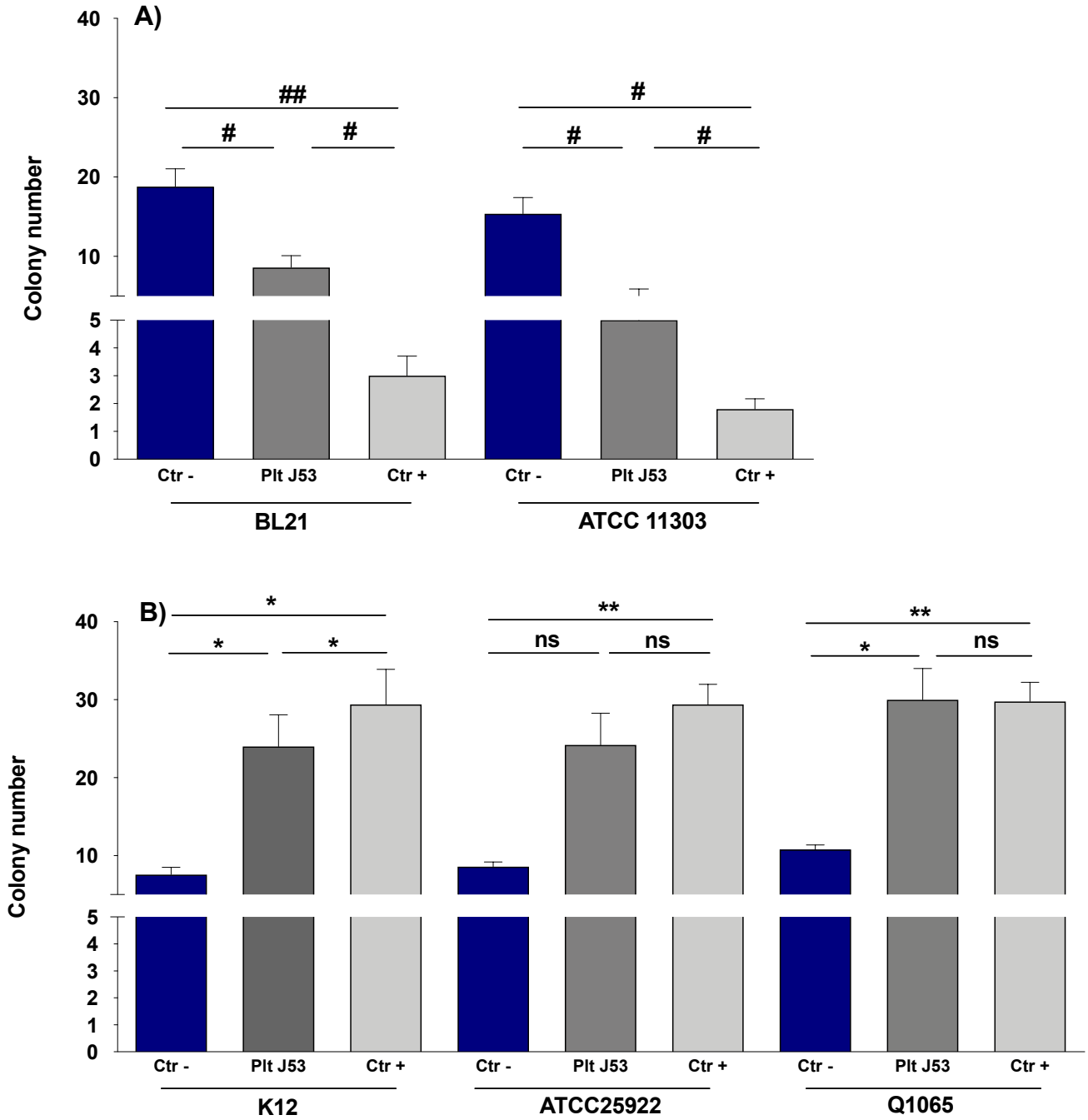

Figure 2. Effect of platelet supernatant on the growth of *E. coli* strains. Bacteria (20 µL, 3×10^8 CFU) were added to filtered supernatant (180 µL) and incubated together at 37 °C for four hours while being rotated. (**A**) Supernatant effect of *E. coli* platelet-sensitive strains BL21DE3 and ATCC11303 ($n = 5$). (**B**) Supernatant effect of *E. coli* platelet-resistant strains K12, ATCC 25922 and Q1065 ($n = 5$). Ctr−: bacteria alone (blue column); Plt J53: bacteria incubated with supernatant of platelets stimulated by J53 (dark grey column); Ctr+: bacteria incubated with supernatant of platelets treated by TRAP (light grey column). Bars represent Mean with SD. *: significant increase; #: significant decrease, ns: non-significant. Significant differences between the two groups were determined by Bonferroni test preceded by two-way ANOVA. * and #: $p < 0.05$, ** and ##: $p < 0.01$, ns: $p > 0.05$.

3.3. The Effect of E. coli Strains on Platelet Activation

In order to assess whether *E. coli* strains induce platelet activation, P-selectin was measured using flow cytometry. After one hour of co-incubation, five strains (ATCC11303, J53, BL21DE3, K12 and ATCC25922) significantly increased P-selectin compared to the

controls (platelets alone). All of the other strains tested did not increase the expression of P-selectin. Significant differences between platelets alone and platelets treated by TRAP as well as between platelets alone and platelets infected with bacteria were determined using the two-tailed, paired Student's *t*-test. Statistical significance was set at $p < 0.05$ (Table 2).

Table 2. Mean Fluorescence Intensity (MFI) percentage of P-selectin expression of platelets infected with *E. coli* strains.

Mean ± SD of MFI % of Platelets Stimulated with *E. coli* Strains and TRAP			
***E. coli* Strains**	**Mean SD of Plt-*E. coli***	***p*-Value Plt-*E. coli* Compared to Plt**	***p*-Value Summary of Plt-TRAP Compared to Plt**
ATCC11303	124.9 ± 14.3	0.007	**
J53	190.7 ± 40.5	0.017	**
BL21DE3	134.5 ± 22.5	0.026	**
K12	106.3 ± 4	0.024	**
ATCC25922	109.1 ± 5.4	0.019	**
LH1	104.2 ± 17.9	-	**
LH30	96.5 ± 7.4	-	**
Q1065	99.4 ± 9.4	-	*
Q1066	102.3 ± 7.8	-	*
Q6269	100.4 ± 9	-	*

Plt: Platelets alone (100%). Plt-*E. coli*: platelets stimulated with *E. coli* strains. Plt-TRAP: platelets treated by TRAP. Percentage of P-Selectin expression in treated and stimulated platelets was calculated by the following equation: (MFI of platelets infected with bacteria) × 100/(MFI of platelets alone).*: $p < 0.05$, **: $p < 0.01$.

The Pearson test was applied to test the correlation between colony count values and the MFI percentage of the different strains in co-incubation with platelets. A negative significant correlation was obtained (Pearson r = −0.6795, *p*-value = 0.0307).

4. Pangenome Analysis

In order to better understand the difference in platelet activation between the *E. coli* strains as previously shown, a pangenome analysis was performed (Figure 3). This study was designed to identify the difference between platelet-activating and non-platelet-activating strains. We were particularly interested in genes present in only one or the other groups of bacteria. In the non-platelet-activating group that consisted of *E. coli* strains LH1, LH30, Q1065, Q1066 and Q6269, no genes shared by all strains were found. In contrast, in the platelet-activating group, six common genes were identified. Of them, two were annotated as elongation factor Tu (elongation factor Tu 1 and 2), two others were annotated as transposase (IS3 family transposase IS911, IS4 family transposase IS4), another was annotated as Outer membrane porin protein OmpD, and the last was annotated as a lactose phosphotransferase system repressor. None of these functions showed a direct link to platelet activation. Furthermore, the analysis of the classification of strains following the pangenome study did not indicate a clustering of bacteria according to platelet activation. Indeed, the clustering put *E. coli* strains BL21 and ATCC11303 in a first group and *E. coli* strains J53, K12 and ATCC25922 in the second (Figure 3).

Thus, the bioinformatic analyses did not identify a gene or a cluster of genes as being at the origin of the difference in platelet activation.

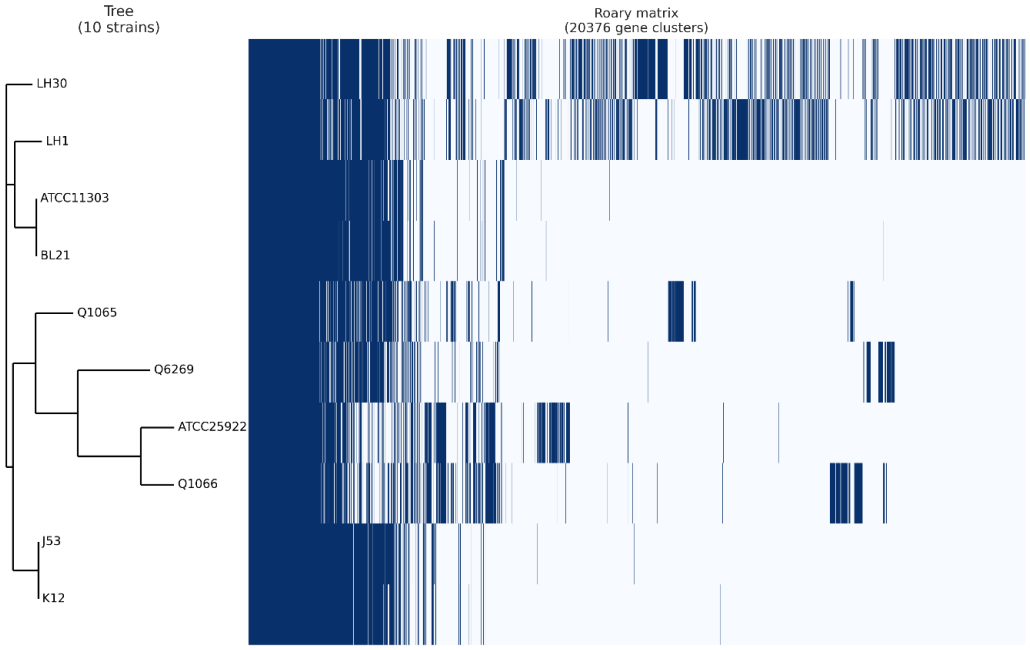

Figure 3. Visualization of pangenome analysis by Roary software of 10 *E. coli* strains. Pangenome analysis of the ten *E. coli* strains using Roary software. Whole genomes of the strains were clustered according to the presence/absence of core genes. Blue: presence of gene, white: absence of gene.

5. O-antigen Strain Serotyping

We investigated the difference in behavior between two genetically related strains, K12 and its mutant strain J53. Using the Genome Mapper of the EcoCyc database (ecocyc.org) and searching for genes coding for proteins involved in the biosynthesis of the O antigen, and thus in the formation of LPS, we found that the *wbbL* gene had an IS5 insertion in its sequence, making it non-functional, and thus able to modify the structure of LPS (Figure 4).

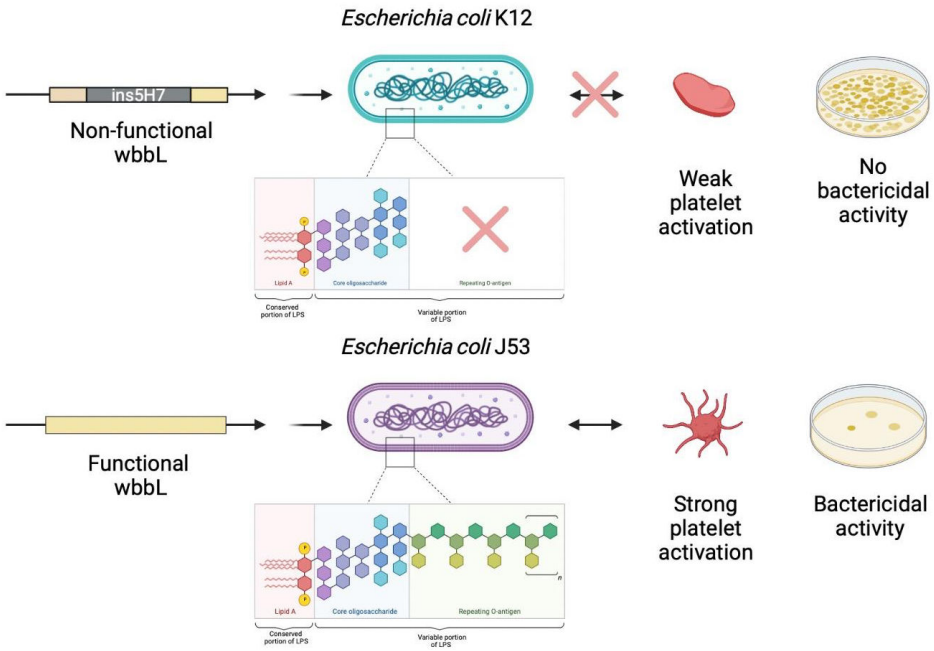

Figure 4. Comparison between the O-antigen biosynthesis cluster of K12 and J53. Comparison between the O-antigen biosynthesis cluster of K12 and its mutant J53. The *E. coli* K12 strain lacks O-antigen, secondary to the presence of mutations, including an IS5 insertion in the gene cluster involved in O-antigen biosynthesis (created with BioRender.com, accessed on 27 April 2022).

6. Discussion

In this study, we evaluated the consequences of interactions between platelets and ten strains of *Escherichia coli*. Our results showed that platelets had an antibacterial activity on three laboratory strains among the ten tested. Genomic comparison of two strains with different susceptibility profiles revealed a difference in the genomic cluster coding for the O-antigen.

To our knowledge, few studies have evaluated the bactericidal effect of platelets against *Escherichia coli* strains [19,36]. Moreover, the originality of our study resides in the fact that we tested a large panel of strains, including both laboratory and clinical strains, which are further distinguished by their colistin-resistance profile.

The bactericidal effect of platelets has been previously tested in two studies, where two of the five colistin-sensitive laboratory strains selected in our study were used. Our results confirmed that *E. coli* ATCC1130 growth inhibition was inhibited by platelets, as previously demonstrated by Tohidnezhad et al. [37]. Moreover, as described by Cieślik-Bielecka et al., we confirmed that the growth of the *E. coli* ATCC25922 strain was not inhibited by platelets [38]. The concordance of these results validated the choice of our experimental model.

Among the ten strains tested, only three were sensitive to platelet bactericidal activity. The interaction of platelets with these strains induced platelet activation responsible for a secretion process of platelet granule content, as evidenced by the increased expression of P-selectin. The bactericidal activity observed is probably due to the action of microbicidal platelet peptides released, since the same effect is observed with the supernatant of activated platelets. We have already described this mechanism for *Staphylococcus aureus* [13]. These three strains of *E. coli*, which were sensitive to platelets, are all laboratory strains that do not express resistance mutations and are not responsible for human infectious pathologies. Interestingly, the two other *E. coli* laboratory strains tested (K12 and ATCC 25922) also induced platelet activation. Regarding this last point, our results are in line with those of Fejes et al., who also demonstrated that the K12 strain induces an increase in P-selectin and CD63 [22]. However, they are insensitive to platelet bactericidal activity. This lack of effect could be the consequence of weak platelet activation induced by these strains, as we have demonstrated a negative correlation between the inhibitory effect of platelets and the activating effect of *E. coli* strains, which means the more the strains increase platelet granule release, the less bacterial growth decreases in the presence of platelets. However, regarding the *E. coli* K12 strain, Palankar et al. found that the bactericidal effect was only obtained with the LPS mutant *E. coli* strains, but not on the wild strain (K12) [36].

Previous studies have suggested that the difference between the profiles of the *E. coli* strains regarding platelets can be explained by the existence of two types of LPS and their interactions with immune cells, which may be the same mechanism for platelets [22]. Indeed, "rough" LPS could activate a wider range of cells with greater efficiency compared to the "smooth" form [39]. Macrophages have been shown to be able to respond to "rough" LPS and lipid A, but not to "smooth" LPS. Furthermore, the "smooth" form requires CD14 to activate immune cells [40].

When looking at the overall effect of each strain on the different parameters studied (platelet activation and inhibitory effect), different platelet interaction profiles can be determined. It could be hypothesized that these profiles are dependent on the structure of the LPS O-antigen of each *E. coli* strain. Indeed, it has long been shown that platelets expose TLR-4 on their surface, which is involved in the recognition of LPS [41]. It could be hypothesized that structural abnormalities of LPS might induce an alteration of the phenomenon of recognition of bacterial structural patterns through this TLR-4.

Resistance to colistin can also be implicated in generating a difference in responses to platelets, since LPS more precisely lipid A, represents the target of colistin, which is also the principal element that interacts with the platelet receptor TLR4. A possible modification of LPS can cause a defect in the interaction with platelets, which can lead to platelet non-activation and resistance to platelet peptides, especially cationic ones which share

several characteristics with colistin, namely their polarity and their modes of action [42,43]. Moreover, the 5 colistin resistant strains could not activate the platelets and they are all resistant to the platelets, which can constitute a problem during an *E. coli* infection which should not be neglected.

Conclusions could not be drawn from the pangenome results as to the gene(s) responsible for platelet activation or strain sensitivity toward platelets. We therefore turned to the prediction of the O-antigen type and were mainly interested in the comparison between K12 and its mutant J53, which have distinct profiles based on bacterial growth results. The *E. coli* K12 strain is known to lack O-antigen, secondary to the presence of mutations, including an IS5 insertion (Figure 3) in the gene cluster involved in O-antigen biosynthesis, as well as core LPS [44]. From this reference K-12 strain, a mutant was developed (K-12 W3110) by transposing the *rfb* gene cluster from the WG1strain. This K-12 W3110 strain was shown to express an O16-type O-antigen [45]. The J53 strain, which is derived from K12, has a deletion of IS5 in this gene cluster, which may indicate that this J53 strain has a functional O-antigen [46]. Our data, as shown above, demonstrate that the K12 and J53 strains seem to have an opposite profile in terms of platelet bactericidal effect. This could be explained by the changes in the structure of the LPS, which is support by data from the genetic database. This hypothesis, that platelet activation is dependent upon the O-antigen carried by bacteria, can also be supported by the fact that TLR-4-dependent signaling pathways leading to platelet activation and aggregation have been shown to exist [47].

In summary, based on data from the literature and our results, we hypothesize that the platelet activation and the antibacterial effect against *E.coli* originate from the same mechanism of action, potentially the bacterial LPS, and that a variability or a structural modification of the LPS, leads to both a defect in platelet activation and resistance to platelet peptides [48]. We did not confirm the hypothesis that this difference in behavior could be related to colistin resistance, because 2 colistin sensitive strains have the same profile as colistin resistant strains but we are convinced that this notion of cross-resistance should be more emphasized.

We believe that these new observations are worth sharing. However, we are aware that further studies, involving proteomic and genomic analyses, are needed to better explain the molecular basis of the differential behavior of *E. coli* strains towards platelets.

7. Conclusions

In conclusion, our work evaluated the bactericidal effect of platelets on ten *E. coli* strains with different characteristics (clinical strains/laboratory strains, colistin resistance profile). On one hand, we have demonstrated a correlation between platelet activation induced by *E coli* and bactericidal activity. On the other hand, our preliminary data, obtained by studying the structure of the O antigen of two laboratory strains, suggest that modification of the O antigen would be responsible for this sensitivity to the bactericidal activity of platelets.

Since researchers have described the interaction between platelets and *E. coli* as a complex interaction, and the fact that all of the clinical strains tested in our study showed resistance to platelet peptides and that their bacterial growth is increased in the presence of platelets, it is important to further investigate the mechanisms of this interaction. Further work should be conducted by testing other clinical strains and by targeting other phenomena such as platelet aggregation to fully understand and identify all the factors involved in this interaction. This will be necessary in order to establish a clinical model of sepsis and HUS for subsequent optimal use of existing drugs and possible development of new drugs.

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References

1. Nurden, A. Platelets, Inflammation and Tissue Regeneration. *Thromb. Haemost.* **2011**, *105*, S13–S33. [CrossRef] [PubMed]
2. Thomas, M.R.; Storey, R.F. The Role of Platelets in Inflammation. *Thromb. Haemost.* **2015**, *114*, 449–458. [CrossRef] [PubMed]
3. Kerrigan, S.W.; Douglas, I.; Wray, A.; Heath, J.; Byrne, M.F.; Fitzgerald, D.; Cox, D. A Role for Glycoprotein Ib in Streptococcus Sanguis-Induced Platelet Aggregation. *Blood* **2002**, *100*, 509–516. [CrossRef] [PubMed]
4. O'Seaghdha, M.; van Schooten, C.J.; Kerrigan, S.W.; Emsley, J.; Silverman, G.J.; Cox, D.; Lenting, P.J.; Foster, T.J. Staphylococcus Aureus Protein A Binding to von Willebrand Factor A1 Domain Is Mediated by Conserved IgG Binding Regions. *FEBS J.* **2006**, *273*, 4831–4841. [CrossRef]
5. Cox, D.; Kerrigan, S.W.; Watson, S.P. Platelets and the Innate Immune System: Mechanisms of Bacterial-Induced Platelet Activation. *J. Thromb. Haemost. JTH* **2011**, *9*, 1097–1107. [CrossRef]
6. Hamzeh-Cognasse, H.; Damien, P.; Chabert, A.; Pozzetto, B.; Cognasse, F.; Garraud, O. Platelets and Infections—Complex Interactions with Bacteria. *Front. Immunol.* **2015**, *6*, 82. [CrossRef]
7. Kerrigan, S.W. Platelet Interactions with Bacteria. In *The Non-Thrombotic Role of Platelets in Health and Disease*; Kerrigan, S.W., Moran, N., Eds.; InTech: Dublin, Ireland, 2015.
8. Rose, P.E.; Armour, J.A.; Williams, C.E.; Hill, F.G. Verotoxin and Neuraminidase Induced Platelet Aggregating Activity in Plasma: Their Possible Role in the Pathogenesis of the Haemolytic Uraemic Syndrome. *J. Clin. Pathol.* **1985**, *38*, 438–441. [CrossRef]
9. Ghosh, S.A.; Polanowska-Grabowska, R.K.; Fujii, J.; Obrig, T.; Gear, A.R.L. Shiga Toxin Binds to Activated Platelets. *J. Thromb. Haemost.* **2004**, *2*, 499–506. [CrossRef]
10. Proulx, F.; Seidman, E.G.; Karpman, D. Pathogenesis of Shiga Toxin-Associated Hemolytic Uremic Syndrome. *Pediatr. Res.* **2001**, *50*, 163–171. [CrossRef]
11. Flaumenhaft, R. Platelet Alpha-Granules: Basic Biology and Clinical Correlates. *Blood Rev.* **2009**, *23*, 177–189. [CrossRef]
12. Portier, I.; Campbell, R.A. Role of Platelets in Detection and Regulation of Infection. *Arterioscler. Thromb. Vasc. Biol.* **2021**, *41*, 70–78. [CrossRef] [PubMed]
13. Hannachi, N.; Fournier, P.-E.; Martel, H.; Habib, G.; Camoin-Jau, L. Statins Potentiate the Antibacterial Effect of Platelets on *Staphylococcus Aureus*. *Platelets* **2021**, *32*, 671–676. [CrossRef] [PubMed]
14. Kraemer, B.F.; Campbell, R.A.; Schwertz, H.; Cody, M.J.; Franks, Z.; Tolley, N.D.; Kahr, W.H.A.; Lindemann, S.; Seizer, P.; Yost, C.C.; et al. Novel Anti-Bacterial Activities of β-Defensin 1 in Human Platelets: Suppression of Pathogen Growth and Signaling of Neutrophil Extracellular Trap Formation. *PLoS Pathog.* **2011**, *7*, e1002355. [CrossRef] [PubMed]
15. Yeaman, M.R. Platelets in Defense against Bacterial Pathogens. *Cell. Mol. Life Sci. CMLS* **2010**, *67*, 525–544. [CrossRef] [PubMed]
16. Akashi, S.; Saitoh, S.; Wakabayashi, Y.; Kikuchi, T.; Takamura, N.; Nagai, Y.; Kusumoto, Y.; Fukase, K.; Kusumoto, S.; Adachi, Y.; et al. Lipopolysaccharide Interaction with Cell Surface Toll-like Receptor 4-MD-2: Higher Affinity than That with MD-2 or CD14. *J. Exp. Med.* **2003**, *198*, 1035–1042. [CrossRef] [PubMed]
17. Park, B.S.; Lee, J.-O. Recognition of Lipopolysaccharide Pattern by TLR4 Complexes. *Exp. Mol. Med.* **2013**, *45*, e66. [CrossRef]
18. Vallance, T.M.; Zeuner, M.-T.; Williams, H.F.; Widera, D.; Vaiyapuri, S. Toll-Like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis. *Mediat. Inflamm.* **2017**, *2017*, 9605894. [CrossRef]
19. Moriarty, R.D.; Cox, A.; McCall, M.; Smith, S.G.J.; Cox, D. *Escherichia Coli* Induces Platelet Aggregation in an FcγRIIa-Dependent Manner. *J. Thromb. Haemost.* **2016**, *14*, 797–806. [CrossRef]
20. Watson, C.N.; Kerrigan, S.W.; Cox, D.; Henderson, I.R.; Watson, S.P.; Arman, M. Human Platelet Activation by Escherichia Coli: Roles for FcγRIIA and Integrin AIIbβ3. *Platelets* **2016**, *27*, 535–540. [CrossRef]
21. Matus, V.; Valenzuela, J.G.; Hidalgo, P.; Pozo, L.M.; Panes, O.; Wozniak, A.; Mezzano, D.; Pereira, J.; Sáez, C.G. Human Platelet Interaction with E. Coli O111 Promotes Tissue-Factor-Dependent Procoagulant Activity, Involving Toll like Receptor 4. *PLoS ONE* **2017**, *12*, e0185431. [CrossRef]
22. Fejes, A.V.; Best, M.G.; van der Heijden, W.A.; Vancura, A.; Verschueren, H.; de Mast, Q.; Wurdinger, T.; Mannhalter, C. Impact of Escherichia Coli K12 and O18:K1 on Human Platelets: Differential Effects on Platelet Activation, RNAs and Proteins. *Sci. Rep.* **2018**, *8*, 16145. [CrossRef] [PubMed]

23. Ståhl, A.; Svensson, M.; Mörgelin, M.; Svanborg, C.; Tarr, P.I.; Mooney, J.C.; Watkins, S.L.; Johnson, R.; Karpman, D. Lipopolysaccharide from Enterohemorrhagic Escherichia Coli Binds to Platelets through TLR4 and CD62 and Is Detected on Circulating Platelets in Patients with Hemolytic Uremic Syndrome. *Blood* **2006**, *108*, 167–176. [CrossRef] [PubMed]
24. Cattaneo, M.; Cerletti, C.; Harrison, P.; Hayward, C.P.M.; Kenny, D.; Nugent, D.; Nurden, P.; Rao, A.K.; Schmaier, A.H.; Watson, S.P.; et al. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. *J. Thromb. Haemost. JTH* **2013**, *11*, 1183–1189. [CrossRef] [PubMed]
25. Hadjadj, L.; Riziki, T.; Zhu, Y.; Li, J.; Diene, S.M.; Rolain, J.-M. Study of Mcr-1 Gene-Mediated Colistin Resistance in Enterobacteriaceae Isolated from Humans and Animals in Different Countries. *Genes* **2017**, *8*, 394. [CrossRef]
26. Venter, P.; Lues, J.F.R. Extraction Methods for Lipopolysaccharides from Escherichia Coli ATCC 25922 for Quantitative Analysis by Capillary Electrophoresis. *Int. J. Food Microbiol.* **2003**, *84*, 245–250. [CrossRef]
27. Pacífico, C.; Hilbert, M.; Sofka, D.; Dinhopl, N.; Pap, I.-J.; Aspöck, C.; Carriço, J.A.; Hilbert, F. Natural Occurrence of Escherichia Coli-Infecting Bacteriophages in Clinical Samples. *Front. Microbiol.* **2019**, *10*, 2484. [CrossRef]
28. Serres, M.H.; Gopal, S.; Nahum, L.A.; Liang, P.; Gaasterland, T.; Riley, M. A Functional Update of the Escherichia Coli K-12 Genome. *Genome Biol.* **2001**, *2*, RESEARCH0035. [CrossRef]
29. Yi, H.; Cho, Y.-J.; Yong, D.; Chun, J. Genome Sequence of Escherichia Coli J53, a Reference Strain for Genetic Studies. *J. Bacteriol.* **2012**, *194*, 3742–3743. [CrossRef]
30. Jeong, H.; Kim, H.J.; Lee, S.J. Complete Genome Sequence of Escherichia Coli Strain BL21. *Genome Announc.* **2015**, *3*, e00134-15. [CrossRef]
31. Hannachi, N.; Grac, L.; Baudoin, J.-P.; Fournier, P.-E.; Habib, G.; Camoin-Jau, L. Effect of Antiplatelet Agents on Platelet Antistaphylococcal Capacity: An in Vitro Study. *Int. J. Antimicrob. Agents* **2020**, *55*, 105890. [CrossRef]
32. Seemann, T. Prokka: Rapid Prokaryotic Genome Annotation. *Bioinformatics* **2014**, *30*, 2068–2069. [CrossRef] [PubMed]
33. Page, A.J.; Cummins, C.A.; Hunt, M.; Wong, V.K.; Reuter, S.; Holden, M.T.G.; Fookes, M.; Falush, D.; Keane, J.A.; Parkhill, J. Roary: Rapid Large-Scale Prokaryote Pan Genome Analysis. *Bioinformatics* **2015**, *31*, 3691–3693. [CrossRef] [PubMed]
34. Bessonov, K.; Laing, C.; Robertson, J.; Yong, I.; Ziebell, K.; Gannon, V.P.J.; Nichani, A.; Arya, G.; Nash, J.H.E.; Christianson, S. ECTyper: In Silico Escherichia Coli Serotype and Species Prediction from Raw and Assembled Whole-Genome Sequence Data. *Microb. Genom.* **2021**, *7*, 12. [CrossRef] [PubMed]
35. Joensen, K.G.; Tetzschner, A.M.M.; Iguchi, A.; Aarestrup, F.M.; Scheutz, F. Rapid and Easy in Silico Serotyping of Escherichia Coli Isolates by Use of Whole-Genome Sequencing Data. *J. Clin. Microbiol.* **2015**, *53*, 2410–2426. [CrossRef]
36. Palankar, R.; Kohler, T.P.; Krauel, K.; Wesche, J.; Hammerschmidt, S.; Greinacher, A. Platelets Kill Bacteria by Bridging Innate and Adaptive Immunity via Platelet Factor 4 and FcγRIIA. *J. Thromb. Haemost. JTH* **2018**, *16*, 1187–1197. [CrossRef]
37. Tohidnezhad, M.; Varoga, D.; Wruck, C.J.; Podschun, R.; Sachweh, B.H.; Bornemann, J.; Bovi, M.; Sönmez, T.T.; Slowik, A.; Houben, A.; et al. Platelets Display Potent Antimicrobial Activity and Release Human Beta-Defensin 2. *Platelets* **2012**, *23*, 217–223. [CrossRef]
38. Cieślik-Bielecka, A.; Bold, T.; Ziółkowski, G.; Pierchała, M.; Królikowska, A.; Reichert, P. Antibacterial Activity of Leukocyte- and Platelet-Rich Plasma: An in Vitro Study. *BioMed Res. Int.* **2018**, *2018*, 9471723. [CrossRef]
39. Jiang, Z.; Georgel, P.; Du, X.; Shamel, L.; Sovath, S.; Mudd, S.; Huber, M.; Kalis, C.; Keck, S.; Galanos, C.; et al. CD14 Is Required for MyD88-Independent LPS Signaling. *Nat. Immunol.* **2005**, *6*, 565–570. [CrossRef]
40. Huber, M.; Kalis, C.; Keck, S.; Jiang, Z.; Georgel, P.; Du, X.; Shamel, L.; Sovath, S.; Mudd, S.; Beutler, B.; et al. R-Form LPS, the Master Key to the Activation OfTLR4/MD-2-Positive Cells. *Eur. J. Immunol.* **2006**, *36*, 701–711. [CrossRef]
41. Andonegui, G.; Kerfoot, S.M.; McNagny, K.; Ebbert, K.V.J.; Patel, K.D.; Kubes, P. Platelets Express Functional Toll-like Receptor-4. *Blood* **2005**, *106*, 2417–2423. [CrossRef]
42. Andersson, D.I.; Hughes, D.; Kubicek-Sutherland, J.Z. Mechanisms and Consequences of Bacterial Resistance to Antimicrobial Peptides. *Drug Resist. Updates* **2016**, *26*, 43–57. [CrossRef] [PubMed]
43. Hancock, R.E.; Diamond, G. The Role of Cationic Antimicrobial Peptides in Innate Host Defences. *Trends Microbiol.* **2000**, *8*, 402–410. [CrossRef]
44. Jeong, H.; Barbe, V.; Lee, C.H.; Vallenet, D.; Yu, D.S.; Choi, S.-H.; Couloux, A.; Lee, S.-W.; Yoon, S.H.; Cattolico, L.; et al. Genome Sequences of Escherichia Coli B Strains REL606 and BL21(DE3). *J. Mol. Biol.* **2009**, *394*, 644–652. [CrossRef] [PubMed]
45. Bilge, S.S.; Vary, J.C.; Dowell, S.F.; Tarr, P.I. Role of the Escherichia Coli O157:H7 O Side Chain in Adherence and Analysis of an Rfb Locus. *Infect. Immun.* **1996**, *64*, 4795–4801. [CrossRef] [PubMed]
46. Matsumura, Y.; Peirano, G.; Pitout, J.D.D. Complete Genome Sequence of Escherichia Coli J53, an Azide-Resistant Laboratory Strain Used for Conjugation Experiments. *Genome Announc.* **2018**, *6*, e00433-18. [CrossRef] [PubMed]
47. Niklaus, M.; Klingler, P.; Weber, K.; Koessler, A.; Boeck, M.; Kobsar, A.; Koessler, J. The Involvement of Toll-like Receptors 2 and 4 in Human Platelet Signalling Pathways. *Cell. Signal.* **2020**, *76*, 109817. [CrossRef]
48. Baron, S.; Hadjadj, L.; Rolain, J.-M.; Olaitan, A.O. Molecular Mechanisms of Polymyxin Resistance: Knowns and Unknowns. *Int. J. Antimicrob. Agents* **2016**, *48*, 583–591. [CrossRef]

Préambule

Les phénomènes d'activation et d'agrégation plaquettaire constituent les principales conséquences de l'interaction entre les plaquettes et les bactéries. En effet, deux situations peuvent être observées

i) Les bactéries stimulent les plaquettes ce qui induit une activation, la libération du contenu granulaire et la formation des amas plaquettaire. Ce processus induit une sécrétion granulaire et la libération de molécules dotées d'activité bactéricide.

ii) Les bactéries n'activent pas les plaquettes, par conséquent, il n'y aura pas de dégranulation et les bactéries ne seront pas inhibées par les plaquettes.

Différentes souches d'*E. coli* activent les plaquettes et induisent une agrégation (30–33). Ces travaux ont étudié ces mécanismes principalement par cytométrie en flux ou par des techniques classiques d'agrégométrie, mais aucune étude n'a caractérisé ces phénomènes par microscopie électronique (ME).

L'étude des plaquettes par la microcopie électronique date de plus de 70 ans. Les premières études utilisant la ME avaient pour objectif de comprendre la biologie des plaquettes, d'identifier le contenu intra-plaquettaire, d'analyser les changements morphologiques lors de l'activation et le rôle critique des composants cytosquelettiques des plaquettes (48). Depuis, la ME a démontré son intérêt en recherche fondamentale et clinique pour étudier la structure des thrombus mais aussi pour analyser l'ultrastructure des plaquettes dans des modèles cliniques afin de développer de nouvelles méthodes diagnostic (49). Notre équipe maîtrise ces techniques de ME. En effet, des techniques de ME ont été utilisées pour détecter la présence de bactéries sur les valves cardiaques et étudier l'organisation cellulaire des végétations (50).

Dans ce travail, nous avions pour objectif d'étudier la structure des amas plaquettaires induits par des souches d'*E. coli* afin de visualiser l'organisation cellulaire des préparations plaquettes-*E. coli* en utilisant la microscopie électronique à balayage (MEB). Trois différentes préparations de plaquettes-*E. coli* ont été observées entièrement et en coupes ultrafines par MEB.

Nos résultats ont révélé des différences importantes dans l'aspect des plaquettes ainsi que leur colocalisation avec les bactéries. En effet, l'état d'activation plaquettaire en réponse aux souches d'*E. coli* testées est bien illustré dans ce travail. Nous avons démontré qu'il est principalement dépendant de la souche testée. En effet, trois niveaux d'activation ont été observés en fonction de la souche : absence d'activation, activation modérée et hyper activation. Dans les deux premiers cas, le contenu granulaire est conservé à l'intérieur des plaquettes. À

l’opposé, dans le troisième cas les plaquettes sont complètement dégranulées. Les souches activant les plaquettes sont piégées dans le magma plaquettaire. En revanche, les souches qui n'induisent pas d'activation significative sont présentes sur les amas plaquettaires.

Nous avons également visualisé la morphologie des bactéries en utilisant la MEB et la microscopie électronique à transmission (TEM). Les images montrent la présence d’un réseau de reliefs épais qui pourrait protéger la bactérie contre l’effet bactéricide des plaquettes et ainsi expliquer les résultats obtenus.

L’analyse microscopique a permis une visualisation du phénomène d’activation plaquettaire ainsi que l’organisation cellulaire des plaquettes et des bactéries. Les résultats de ce travail ont donc confirmé et complété nos résultats précédents, à savoir que les interactions entre les plaquettes et les souches *d’E. coli* sont dépendantes de la souche utilisée.

Article 3: Microscopic description of platelet aggregates induced by *Escherichia coli* strains

Ezzeroug Ezzraimi, A. ; Baudoin, J.-P. ; Mariotti, A. ; Camoin-Jau, L.

Description microscopique des agrégats de plaquettes induits par les souches *d'Escherichia coli*. *Cellules* ***2022****, 11,* 3495.

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cells

MDPI

Article

Microscopic Description of Platelet Aggregates Induced by *Escherichia coli* Strains

Amina Ezzeroug Ezzraimi [1,2], Jean-Pierre Baudoin [1,2], Antoine Mariotti [1,2,3] and Laurence Camoin-Jau [1,2,3,*]

1 Aix Marseille University Faculté de Médecine t de Pharmacie, IRD, APHM, MEPHI, IHU Méditerranée Infection, 13385 Marseille, France
2 IHU Méditerranée Infection, Boulevard Jean Moulin, 13385 Marseille, France
3 Laboratoire d'Hématologie, Hôpital de la Timone, APHM, Boulevard Jean-Moulin, 13385 Marseille, France
* Correspondence: laurence.camoin@ap-hm.fr; Tel.: +33-4-13-73-24-01; Fax: +33-4-13-73-24-02

Abstract: In addition to their role in haemostasis, platelets are also involved in the inflammatory and antimicrobial process. Interactions between pathogens and platelets, mediated by receptors can lead to platelet activation, which may be responsible for a granular secretion process or even aggregation, depending on the bacterial species. Granular secretion releases peptides with bactericidal activity as well as aggregating factors. To our knowledge, these interactions have been poorly studied for *Escherichia coli* (*E. coli*). Few studies have characterised the cellular organization of platelet-*E. coli* aggregates. The objective of our study was to investigate the structure of platelet aggregates induced by different *E. coli* strains as well as the ultrastructure of platelet-*E. coli* mixtures using a scanning and transmission electron microscopy (SEM and TEM) approach. Our results show that the appearance of platelet aggregates is mainly dependent on the strain used. SEM images illustrate the platelet activation and aggregation and their colocalisation with bacteria. Some *E. coli* strains induce platelet activation and aggregation, and the bacteria are trapped in the platelet magma. However, some strains do not induce significant platelet activation and are found in close proximity to the platelets. The structure of the *E. coli* strains might explain the results obtained.

Keywords: platelets; *Escherichia coli*; platelet clumps; platelet activation; platelet aggregation; microscopy

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1. Introduction

Although platelets have many functions, these fragments of megakaryocytes play an essential role in haemostasis [1,2]. Platelets contain numerous specialised organelles dedicated to various functions related to inflammatory and antimicrobial processes. Platelets can interact with bacteria, which can lead to their activation and aggregation [3,4]. However, these mechanisms depend on several factors, mainly the bacterial species and even the strain studied.

Although a great deal of research has been conducted on interactions between platelets and Gram-positive bacteria [5–7], which can interact with platelets indirectly via von Willebrand Factor (vWF) or directly. *Staphyloccocus aureus*, *Streptococcus gordonii* and *Streptococcus sanguinis* bind directly to platelets by involving proteins (SrpA, GrspB and SrpA respectively) via the platelet GPIbα [7]. The data on Gram-negative bacteria, in particular *Escherichia coli*, remain insufficient to understand the molecular mechanism of these interactions and to understand the factor of variability of the results. This interaction has been shown to be primarily dependent on TLR4 binding with LPS or by FcγRII recruitment [8].

E. coli is involved in sepsis, especially in the elderly and new-borns. Bacteria act directly on the platelets, which can lead to vascular complications with states of immunothrombosis. Therefore, it is very important to study the interaction between platelets and *E. coli* in order to have more knowledge about this interaction and its consequences.

Recently, we studied the antibacterial effect of platelets on different strains of *E. coli*. It was found that this bactericidal activity was strain dependent [9]. We also observed a

correlation between this bactericidal effect and the capacity of strains to induce platelet activation [9]. Indeed, some *E. coli* strains induced platelet activation, a result that brought into question the platelet aggregation capability of these strains [8,9].

Electron microscopy (EM) has been a crucial tool in the study of platelet biology and thrombosis for more than 70 years [10–13]. We previously used scanning electron microscopy (SEM) to characterise the platelet-bacteria aggregates for different bacteria species, namely *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus sanguinis*. [6]. The aim of this study was to describe, with a large panel of SEM and transmission electron microscopy (TEM) techniques, the morphology and ultrastructure of platelet aggregates induced by three different strains of *E. coli*.

2. Materials and Methods

2.1. Preparation of the Washed Platelets

Blood was drawn by venepuncture in sodium citrate from healthy subjects who were not receiving antibiotic, anti-inflammatory, or anti-platelet drugs. Platelet rich plasma (PRP) was prepared according to the guidelines of the International Society on Thrombosis and Haemostasis (ISTH) [14]. A platelet count was performed using a haematology analyser. The PRP was again centrifuged at 1100× *g* for ten minutes to obtain a platelet pellet that was suspended in phosphate buffered saline (PBS) to obtain a solution of 4×10^9/L. The platelets were then kept at 37 °C in order to prevent activation. The protocol was approved by the ethics committee of the IHU Méditerranée Infection (Reference 2016–002). All subjects gave their written informed consent in accordance with the Declaration of Helsinki.

2.2. Preparation of Bacteria

The strains used in this study were selected from each group based on our previous results [9]. The two main selection criteria are the capacity of the strain to induce platelet activation and the profile toward the platelet inhibitory effect (Table 1).

Table 1. Origins and profiles of tested strains.

Strain	Origin	Platelet Activation	Platelet Bactericidal Effect	O-Antigen Serotyping	Reference
K12	Laboratory strain	+	-	-	[9]
LH30	Clinical isolate	-	-	O8	[9]
J53	Laboratory strain	+	+	O16	[9]

Platelet activation: the capacity of the strain to induce platelet activation; (+): induce platelet activation, (-): do not induce. Platelet bactericidal effect: the capacity of platelets to inhibit bacterial growth; (+): growth inhibition, (-): no growth inhibition.

The strains represent the following profiles: *E. coli* J53, platelet sensitive strain which induces platelet activation; *E. coli* K12, platelet resistant strain which induces platelet activation; and *E. coli* LH30, platelet resistant strain which does not induce platelet activation (Table 1).

Identification was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and the Biotyper database (Bruker, Dresden, Germany). Strains were grown at 37 °C in an overnight culture of Columbia agar +5% sheep blood (bioMérieux, Marcy l'Etoile, France). After 18 h of incubation at 37 °C, the colonies were removed and suspended in 0.9% NaCl medium to obtain the required concentrations: 1×10^8 CFU (colony format units).

2.3. Scanning Electron Microscopy (SEM) of Whole Platelet-Bacteria Aggregates

As previously described [6], 200 µL of living PBS-washed platelets (4×10^8/mL) and of PBS-washed bacteria (10^9 CFU/mL) were mixed for one hour at 37 °C [9], under rotation to avoid the static state and the development of aggregates due to gravity. Cells were then

fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for one hour. After fixation, samples were rinsed three times with 0.1 M sodium cacodylate (five minutes each) to remove any residual fixative. Cells were dehydrated with graded ethanol concentrations: 25% for five minutes; 50% for five minutes; 70% for five minutes; 85% for five minutes; 95% for five minutes (twice); 100% ethanol for 10 min (three times). Following ethanol dehydration, cells were incubated for five minutes in an ethanol/hexamethyldisilazane (HMDS, Sigma Aldrich, USA) (1:2) mixture, then twice in pure HMDS. Between all steps, cells were gently stirred and centrifuged at 1300 rpm. A drop of cells in pure HDMS was deposited on a glass slide and allowed to air dry for 30 min before observation [6,15] Cells were visualised with a TM4000Plus (Hitachi, Tokyo, Japan) scanning electron microscope operated at 10 kV with Back-Scattered Electrons (BSE) detector at magnifications ranging from X200 to X3000.

2.4. SEM of Ultra-Thin Sections of Platelet-Bacteria Aggregates

Cells mixtures were fixed with glutaraldehyde (2.5%) in 0.1 M sodium cacodylate buffer. Resin embedding was microwave-assisted with a PELCO BiowavePro+. Samples were washed with a mixture of 0.2 M saccharose/0.1 M sodium cacodylate and post-fixed with 1% OsO4 diluted in 0.2 M potassium hexa-cyanoferrate (III)/0.1 M sodium cacodylate buffer. After being washed with distilled water, samples were gradually dehydrated by successive baths containing 30% to 100% ethanol. Substitution with Epon resin was achieved by incubations with 25% to 100% Epon resin. Resin was heat-cured for 72 h at 60 °C. Ultrathin 100 nm sections were cut and placed on HR25 300 Mesh Copper/Rhodium grids (TAAB). Sections were contrasted with uranyl acetate and lead citrate according to Reynolds's method [16]. Grids were attached with double-side tape to a glass slide and platinum-coated at 10 mA for 20 s with a MC1000 sputter coater (Hitachi High-Technologies, Japan). Electron micrographs were obtained on a SU5000 scanning electron microscope (Hitachi High-Technologies, Japan) operated in high-vacuum at 7 kV accelerating voltage and observation mode (spot size 30) with BSE detector. The abundance of bacteria in each mixture was determined by measuring the surface occupied by the bacteria using Fiji software with 10 images for each mixture. The criteria used to indicate that the platelets are hyper-activated are: the presence of pseudopodia and the presence of platelet aggregates; moderately activated: the presence of pseudopodia and the absence of platelet aggregates; not activated: presence of intact platelets in lenticular shape, absence of pseudopodia and platelet aggregates. The aggregates presence was determined by measuring the surface occupied by the platelet aggregates using Fiji software with 10 images for each mixture.

2.5. Transmission Electron Microscopy (TEM) of Negatively Stained Bacteria

Samples of pure bacteria were fixed with glutaraldehyde (2.5%) in 0.1 M sodium cacodylate buffer. A drop of fixed bacterial suspension was applied for five minutes to the top of a formvar carbon 400 mesh nickel grid (FCF400-Ni, EMS), which was previously glow discharged. After drying on filter paper, bacteria were immediately stained with aqueous 1% ammonium molybdate (1-800- ACROS, USA) for 10 s. After drying, electron micrographs of negatively stained bacteria were acquired using a Tecnai G2 transmission electron microscope (Thermo-Fischer/FEI) operated at 200 keV equipped with a 4096 × 4096 pixels resolution Eagle camera (FEI).

2.6. SEM of Whole Bacteria

Bacteria were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for at least one hour. After fixation, bacteria were rinsed for one minute with 0.1 M sodium cacodylate. Bacteria were gradually dehydrated with increasing ethanol concentrations: 30%, 50%, 70%, 90%, and 100% (one minute each). Bacteria were incubated for one minute in ethanol/hexamethyldisilazane (HMDS, Sigma Aldrich, USA) with a 1:2 ratio and finally incubated in pure HMDS. Between all previous steps, cells were gently stirred and centrifuged at 5000 rpm. Finally, 100 µL of each bacteria solution was centrifuged on a

cytospin glass slide at 800 rpm for eight minutes. After deposition, bacteria were air-dried for five minutes and slides were platinum sputter-coated for 20 s at 10 mA (Hitachi MC1000). Observations were made using a SU5000 (Hitachi High-Technologies, Tokyo, Japan) SEM with Secondary-Electrons (SE) detector in high-vacuum mode at 1 kV acceleration voltage, observation mode (spot size 30).

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 (86) for Mac OS X (San Diego, CA, USA, www.graphpad.com, accessed on 27 October 2022) Significant differences (for occupied surface and reliefs thickness) between two groups were determined using the two-tailed, paired Student's *t*-test. The statistical significance was set at $p < 0.05$.

3. Results

3.1. SEM of Whole Platelet-Bacteria Mixtures

To describe the spatial relationship between platelets and *E. coli* after mixing, we used BSE-SEM, detecting back-scattered electrons. Analysis was performed on washed platelets from healthy subjects incubated with three strains of *E. coli.*

BSE-SEM of whole platelet-bacteria mixtures showed that in the case of the K12-platelets aggregates (Figure 1A1,A2), platelets were moderately activated and there were a few platelet aggregates, with many bacteria above these aggregates. In LH30-platelets mixtures (Figure 1B1,B2D), there were many bacteria but few platelet aggregates. In contrast, the J53 strain induced a higher activation as seen by the morphology of the few non-aggregated platelets, and a greater aggregation. Many platelet clumps were found but surprisingly, almost no bacteria were detected in this mixture of platelets-J53 (Figure 1C1,C2). The abundance of bacteria was determined by measuring the occupied surfaces by the bacteria in each platelet-bacteria preparation (Figure 2A). The bacteria present in the platelet-J53 preparation were the least abundant compared to the 2 other preparations (significant differences: $p < 0.0001$ between J35 and K12, $p = 0.0029$ between J53 and LH30). Measure of the occupied surfaces by the platelet aggregates in each platelet-bacteria preparation (Figure 2B) have shown a significantly higher surface area in the platelet –J53 preparation compared to the two other preparations (Figure 2B). The results of this part have been summarized in Table 2.

To check whether bacteria were indeed absent from the platelet aggregates after mixing, we next performed resin-embedding and ultra-thin sectioning on platelets-J53 mixtures to access the internal content of the aggregates.

3.2. SEM of Ultra-Thin Sections of Platelet-Bacteria Mixtures

We performed SEM of the ultra-thin sections of the resin-embedded platelets-bacteria aggregates, using the back-scattered electron (BSE) detector, and these images showed that there were differences in the ultrastructural organisation of the different mixtures regarding the strains.

Indeed, platelets and K12 strain *E. coli* bacteria (Figure 3A1,A2) were found to be mixed within the aggregates. Platelets were intact and moderately activated, with no release of granular content. The bacteria were found in significant numbers between the pseudopods of the activated platelets. For LH30, platelets and bacteria were found side-by-side rather than mixed together (Figure 3B1,B2). Although platelets were found intact, as with K12, platelets were not as activated as with the K12 strain, with a few pseudopods and intact granular contents. In contrast, in the case of the J53 strain, the granular content of the platelets was observed to be released extracellularly into an amorphous matrix (Figure 3C1,C2). *E. coli* J53 bacteria were found to be trapped inside this matrix. These latter results explain the absence of J53 bacteria on the surface of the whole platelet aggregates observed by SEM (Figure 1). The results of this part have been summarized in Table 2.

Table 2. Description of whole and sectioned platelets-bacteria aggregates using SEM.

	BSE-SEM of Whole Platelets-Bacteria Aggregates (Figure 1)			BSE-SEM of Ultrathin Sections of Platelets-Bacteria Aggregates (Figure 2)			
Criteria	**Visible Platelets**	**Visible Bacteria**	**Platelet Activation**	**Platelet Integrity**	**Platelet Activation**	**Platelet Granules**	**Bacteria's Location Regarding Platelets**
K12 strain	Yes	Yes	Moderate	Yes	Moderate	Inside platelets	Mixed
LH30 strain	Yes	Yes	No	Yes	No	Inside platelets	Side by side
J53 strain	Moderate	No	NC	Amorphous matrix	High	Among the amorphous matrix	Inside the amorphous matrix

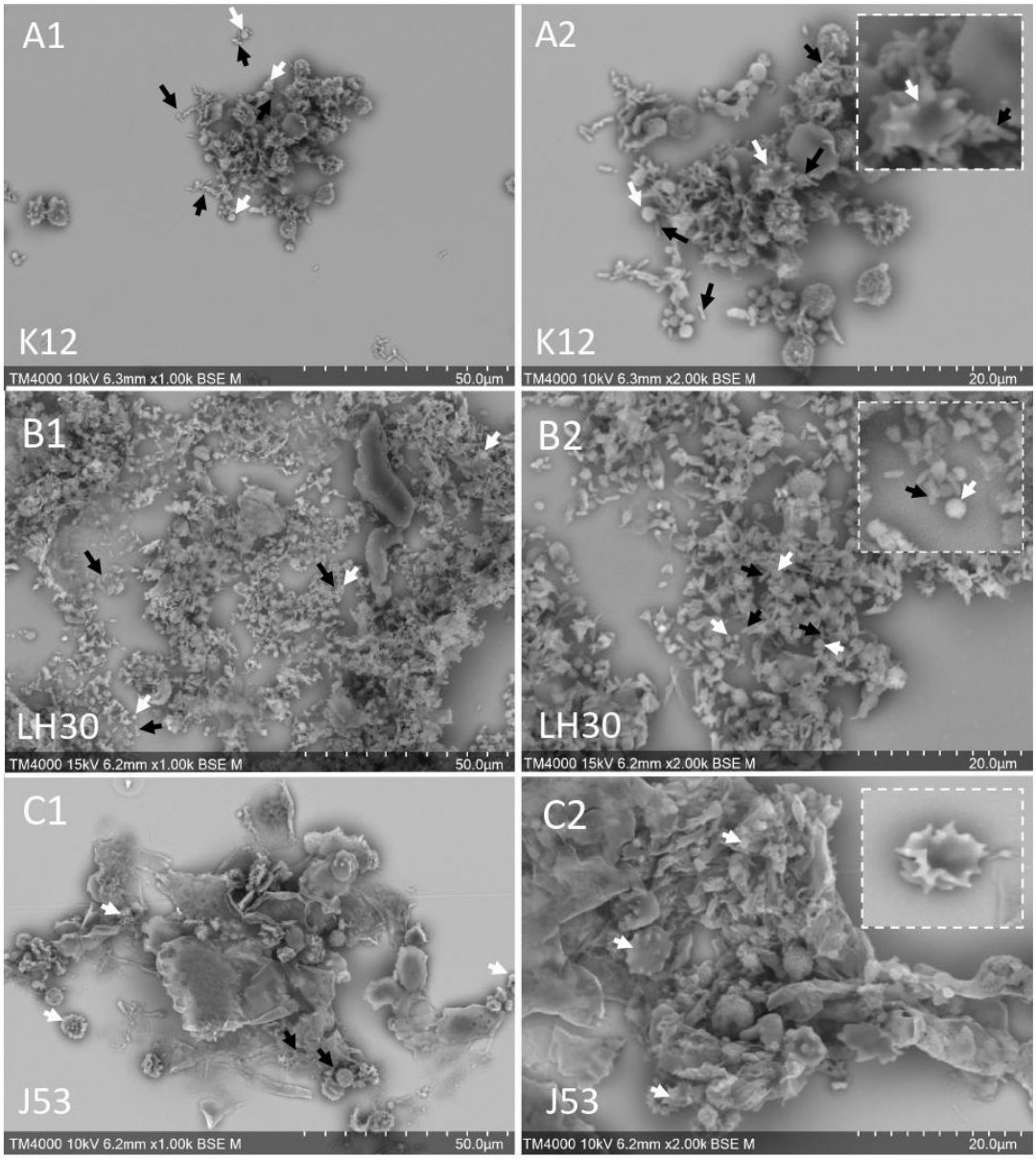

Figure 1. BSE-SEM observation of whole platelets-*E. coli* mixtures. (**A1**,**A2**): Platelets and *E. coli* K12 mixture; (**B1**,**B2**): Platelets and *E. coli* LH30 mixture; (**C1**,**C2**): Platelets and *E. coli* J53 mixture. White arrows: platelets, black arrows: bacteria.

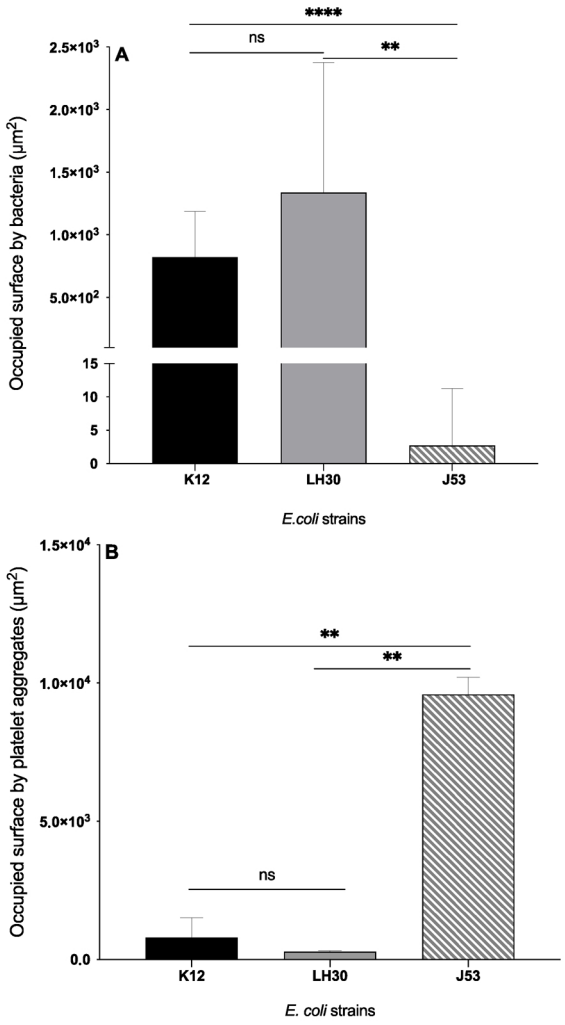

Figure 2. Occupied surface (μm^2) by bacteria (**A**) and platelet aggregates (**B**) for each *E. coli* strain in platelet-bacteria mixtures. (**A**): Bars represent Mean with SD. Black column: occupied surface by *E. coli* K12 ($8.23 \times 10^2 \pm 3.64 \times 10^2$). Grey column: occupied surface by *E. coli* LH30 ($1.34 \times 10^3 \pm 1.04 \times 10^3$). Striped grey column: occupied surface by *E. coli* J53 (2.71 ± 8.57). (**B**): Bars represent Mean with SD. Black column: occupied surface by platelet aggregates in platelets-K12 mixture (795.6 ± 710.5). Grey column: occupied surface by platelet aggregates in platelets-LH30 mixture (281.2 ± 36.71). Striped grey column: occupied surface by platelet aggregates in platelets-J53 mixture (9583 ± 630.7). **,****: significant difference. ns: non-significant. Significant differences between the two groups were determined using the two-tailed, paired Student. ** $p < 0.01$, **** $p < 0.0001$.

To highlight the morphology of platelet-*E. coli* aggregates, we designed this table to be able to compare the aggregates according to the strains, using the two techniques. In order to describe these aggregates, we chose the following criteria: the visibility of platelets and bacteria, the aspect of platelets, platelet activation, the aspect of platelet granules and the colocalisation of bacteria and platelets. NC: non conclusive.

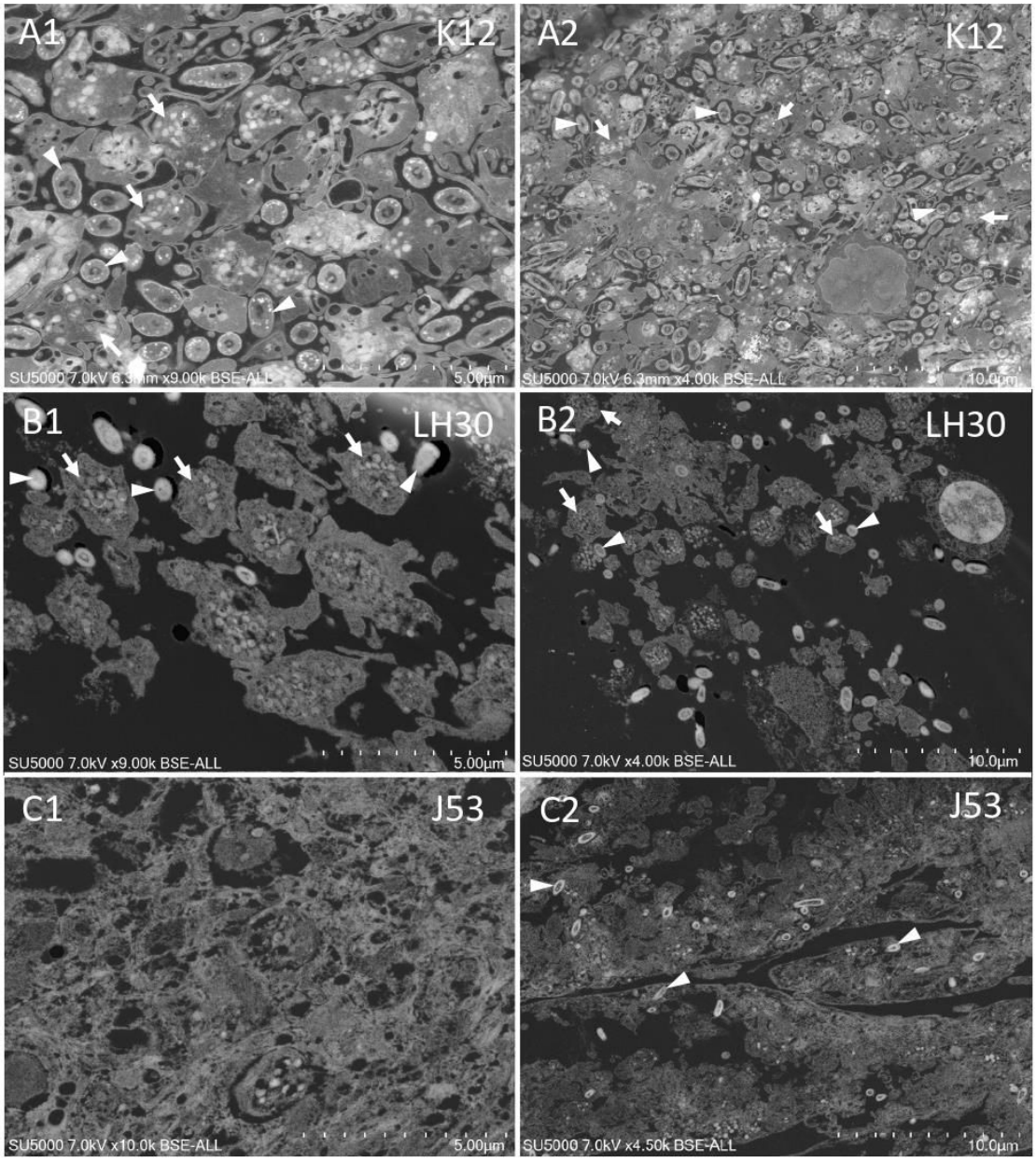

Figure 3. BSE-SEM observation of ultra-thin sections of platelets-*E. coli* mixtures. (**A1**,**A2**): Platelets and *E. coli* K12 mixture, (**B1**,**B2**): Platelets and *E. coli* LH30 mixture; (**C1**,**C2**): Platelets and *E. coli* J53 mixture. White arrows: platelets, white arrowheads: bacteria.

3.3. Electron Microscopy of Bacteria

In order to understand whether ultrastructural differences between the three *E. coli* bacteria strains could explain their respective behaviour regarding platelet aggregation, we performed an in-depth electron microscopy analysis on the cellular level.

First, we analysed the ultrastructure of the bacteria in ultra-thin sections of platelets-bacteria aggregates (Figure 4). We found that for K12 bacteria, the cell wall was regular and attached to the periplasm, and that bacteria possessed electron-dense bodies within an electron-lucent periplasm (Figure 4A1,A2). For LH30 and J53 bacteria, we found close ultrastructures, with irregular or sinuous cell walls for LH30 and J53 bacteria, respectively (Figure 4B1,B2,C1,C2). LH30 and J53 cell walls were found to be detached from an electron-dense periplasm, with a more pronounced detachment for LH30 (Figure 4B2,C2).

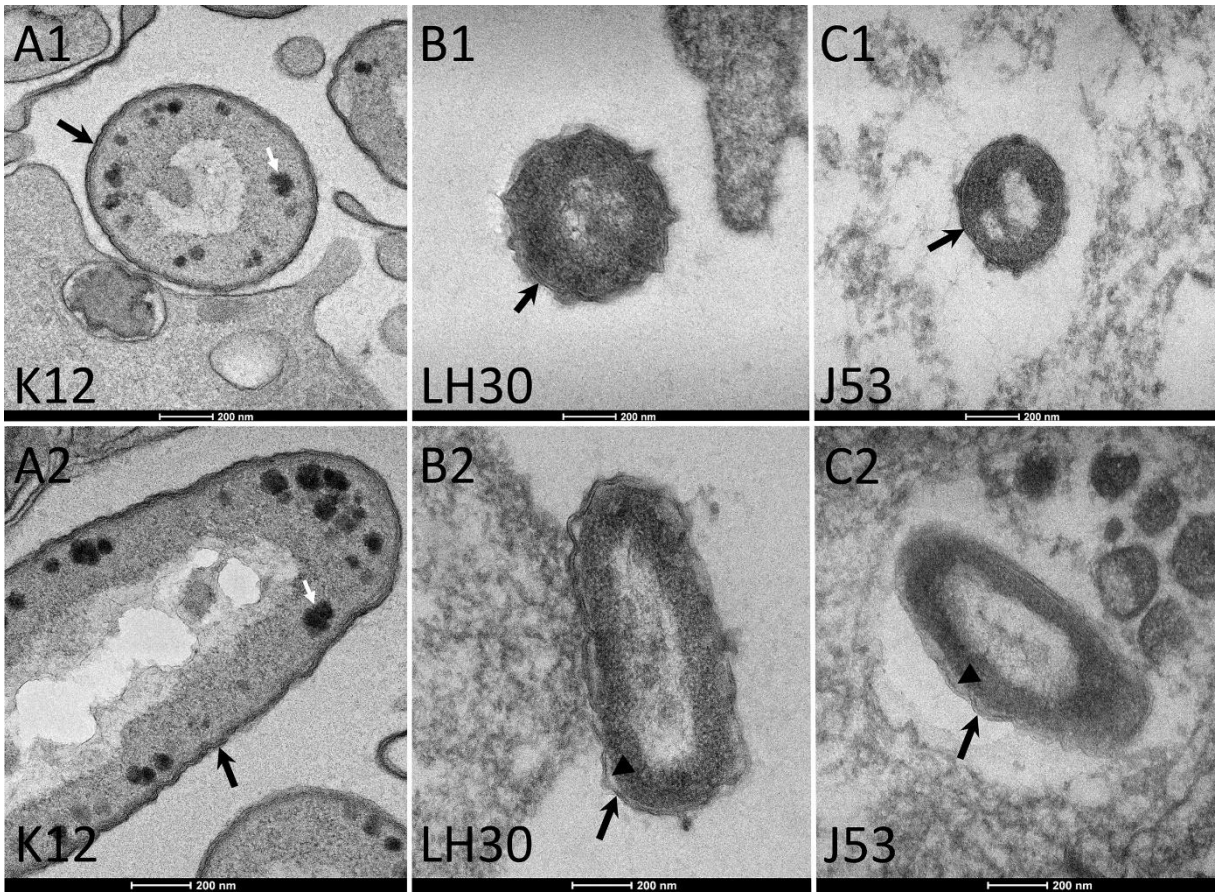

Figure 4. BSE-SEM observation of ultrathin sections of bacteria within the platelet aggregates. (**A1**,**A2**): K12 *E. coli* transversal and longitudinal sections, respectively. (**B1**,**B2**): LH30 *E. coli* transversal and longitudinal sections, respectively. (**C1**,**C2**): J53 *E. coli* transversal and longitudinal sections, respectively. Black arrows: cell wall; white arrows: electron dense bodies; arrowheads: free space between cell wall and periplasm.

Secondly, we analysed whole bacteria by TEM and SEM. When negatively stained and imaged by TEM, the three *E coli* bacteria strains had an elongated shape and J53 strain presented flagella (Figure 5A1,B1,C1). To better describe the surface of the cells, we next performed SEM of the whole bacteria using the secondary electrons (SE) detector. The results of this part have been summarized in Table 3.

Using SE-SEM, we observed that K12 and J53 *E. coli* bacteria possessed a 'rough' surface, composed of a complex network of many thin surface reliefs (Figure 5A2,C2; 14 cells analysed). Measurement of membrane thickening was performed using Fiji software with X bacteria analysed for each strain. In contrast, the surface of LH30 *E coli* bacteria was 'smooth', with a simpler network of larger surface reliefs (Figure 4B2; 14 cells analysed). The thickness of these bacterial reliefs surface was measured, and the results were analysed statistically. The analysis showed that indeed the J53 strain has reliefs that are less thick (47.34 µm ± 18.23) compared to the two other strains (107.8 µm ± 26.81 and 115.2 µm ± 43.04 for K12 and LH30 respectively. p = 0.001 and p = 0.0008 for K12 and LH30 respectively. No significant difference was observed between K12 and LH30 strains). These SEM images also confirmed the presence of flagella on the J53 strain *E. coli* bacteria (not shown) and were used for measuring bacteria dimensions. The average dimensions of the bacteria were 2854 ± 961 nm length and 826 ± 71 nm width for K12 (n = 14), 2570 ± 731 nm length and 1073 ± 121 nm width for LH30 (n = 14) and 2931 ± 483 nm length and 917 ± 329 nm width for J53 (n = 14). No significant differences were observed

between strains regarding length and width. The results of this part have been summarized in Table 3.

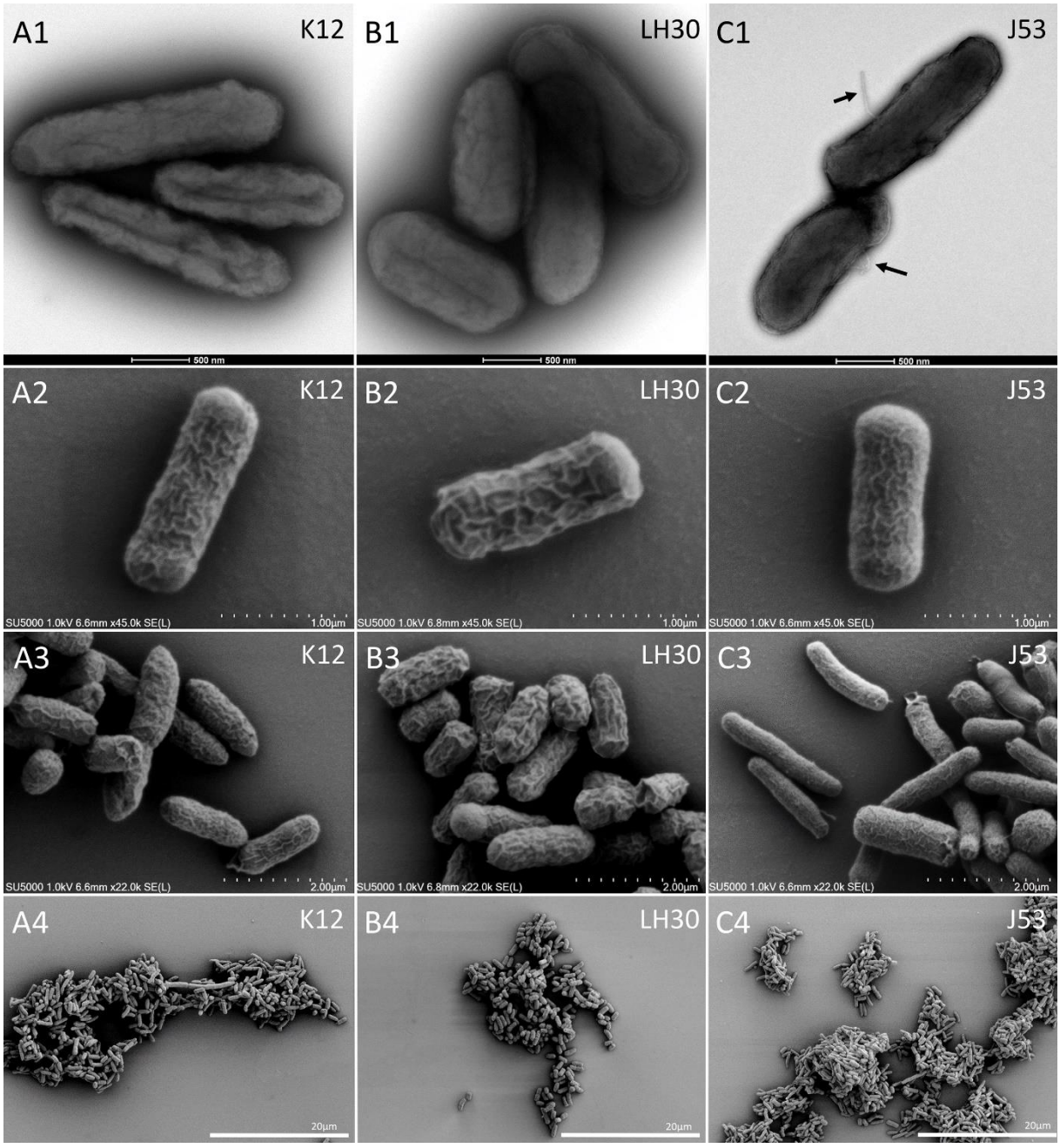

Figure 5. TEM and SE-SEM observation of the whole bacteria strains. (**A1**,**B1**,**C1**): TEM negative staining of K12, LH30 and J53 *E. coli* bacteria, respectively. Arrows in C1 point to flagella. (**A2**–**A4**): Secondary electrons (SE)-SEM of whole K12 *E. coli* bacteria. (**B2**–**B4**): SE-SEM of whole LH30 *E. coli* bacteria. (**C2**–**C4**): SE-SEM of whole J53 *E. coli* bacteria.

Table 3. Bacteria morphology.

E. coli Strain / Technique	BSE-SEM Ultrathin Sections (Figure 3)	TEM Negative Staining (Figure 4)	SE-SEM (Figure 4)
K12	Regular cell wall, attached to periplasm, electron-dense bodies	Elongated shape	Thin surface reliefs
LH30	Irregular shaped cell wall, detached from periplasm, electron-dense periplasm	Elongated shape	Thick surface reliefs
J53	Sinuous cell wall, detached from periplasm, electron-dense periplasm	Elongated shape ± flagella	Thin surface reliefs

4. Discussion

This study described the consequences of the interaction between platelets and three strains of *Escherichia coli* using complementary electron microscopy techniques. Our overall results show that the appearance of aggregates and the colocalisation of bacteria and platelets are strain-dependent. Structural analysis by electron microscopy of the strains could explain our results. This structural variability of the platelet-*E. coli* mixture confirmed our previous results and complements them [9].

Few studies have used fluorescence microscopy to describe the colocalisation of platelets and *E. coli* [12,13]. To our knowledge, none have characterised platelet-*E. coli* mixtures by electron microscopy. The SEM study allowed the analysis of the activation state of the platelets and in particular the granular secretion process. Three platelet activation profiles were obtained depending on the strain tested. Platelet activation would, therefore, be strain dependent, as previously demonstrated by Watson on a reduced panel of two strains [17]. The study of platelet-LH30 mixtures shows the persistence of intra-platelet granules, demonstrating the absence of an activating effect of this strain on platelets, in agreement with the flow cytometry results obtained previously [9]. LH30 is a clinical and colistin-resistant isolate. However, this profile did not allow us to explain the observed patterns. We demonstrated in previous work that *E. coli*-induced platelet activation is independent of the response to colistin [9]. The K12 strain induced moderate activation, as previously observed by Fejes et al. and by our team [9,18]. In contrast, a strong activation, responsible for the formation of an aggregate, was detected for the J53 mixtures. This is both surprising and interesting, because the J53 strain is a mutant of K12.

Among the analytical criteria of our study, we were interested in the number of bacteria present in the mixtures. Comparative SEM analysis of the three platelet-bacteria mixtures shows that the abundancy of bacteria present on the platelet-*E. coli* mixtures is variable depending on the strain tested. It can be hypothesised that the number of bacteria found is a consequence of the bactericidal activity of the platelets. Indeed, platelet activation leads to the release of granular content and, in particular, the release of platelet microbicidal peptides (PMPs), which have bactericidal activity on certain strains. We have previously shown that there is an inverse correlation between the activation state induced by the strains tested and their bactericidal power [9]. This explains why high bacterial abundancy was observed with strain LH30, which did not induce platelet activation, as shown by the persistence of intra-platelet granules. In contrast, in the presence of strain J53, which was responsible for strong aggregation, no bacteria were observed in the SEM images. Whole mount analysis allowed us to detect bacteria trapped in the platelet magma and in low abundancy.

In order to understand the difference in behaviour of the two strains, we analysed the ultrastructure of the bacteria when mixed with platelets in ultra-thin sections by BSE-SEM. The presence of electron-dense bodies in the periplasm of strain K12 was detected which, according to the literature, may be deposits of polyphosphates formed as a result of a defect

in LPS synthesis [19]. These images may illustrate that the K12 strain has a deletion of a mobile IS5 element in the WbbL gene involved in O-antigen biosynthesis [20], meaning that the two strains do not have the same form of LPS. Our hypothesis is that the contrasting behaviour of the two strains toward platelets could be linked to the difference in the LPS form, more precisely antigen-O. Furthermore, this hypothesis is also supported by the fact that it has been shown that platelet TLR-4 is involved in the recognition of LPS [4,21,22]. This TLR-4 signalling pathway is capable of inducing platelet aggregation [23,24]. Our results would help to understand the difference in behaviour between the K12 and J53 strains.

Another point that caught our attention and that we feel should be highlighted is the morphology of bacteria. In fact, the K12 and LH30 strains have a kind of network on their surfaces which is mainly characterised by reliefs. A complex network with thin reliefs was observed on the K12 strain surface, while the LH30 strain has a less complex network on its surface, but with thicker reliefs. These characteristics lead us to suggest that this surface network could be a barrier against the interaction with platelets that prevents activation, and also prevents the effect of PMPs in the case of activated platelets. This difference in structure may explain the difference in behaviour between these two strains.

Our results allow us to better understand our previous work and enable us to understand the heterogeneity of the response of *E. coli* strains against platelets by combining several electron microscopy techniques.

According to our data, the bactericidal mechanism would be of secretory origin. Different scenarios can be distinguished. The platelets are not activated by the bacteria and do not inhibit bacterial growth, which is the case for strain LH30. The absence of platelet activation, probably related to the membrane structure of the strain, which constitutes a physical barrier with the platelets, does not induce the release of PMPs of granular origin. The moderate platelet activation induced by strain K12 is insufficient to induce secretion of granular content. We consider these strains to be platelet resistant. Conversely, the J53 strain strongly activates platelets, responsible for significant platelet aggregation. The bacteria are then trapped in the platelet aggregates and their growth is inhibited. This strain is considered platelet-sensitive. The difference between the behavior of J53 and K12 strains towards platelets is probably related to the absence of a functional O-antigen, which is then reflected in the absence of degranulation".

Since laboratory strains generally behave differently from clinical or wild-type strains, we will need to confirm our observations on a larger number of clinical strains.

The different strain profiles might have important clinical consequences in patients. We could characterize the functional profile of strains in *Escherichia coli* bacteremia by platelet aggregation methods or by ex silico serotyping. Analysis of these data in a prospective study in patients with *Escherichia coli* bacteremia could allow us to validate the clinical relevance of these data.

Author Contributions: A.E.E., A.M., L.C.-J. and J.-P.B. designed the protocol, J.-P.B. ensured technical microscopy set up. A.E.E., A.M. and J.-P.B. performed the experiments. A.E.E., A.M., L.C.-J. and J.-P.B. analysed the results and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of IHU Méditerranée infection (reference 2016-002).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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References

1. Coller, B.S. Historical Perspective and Future Directions in Platelet Research. *J. Thromb. Haemost.* **2011**, *9* (Suppl. S1), 374–395. [CrossRef] [PubMed]
2. Simon, D.; Kunicki, T.; Nugent, D. Platelet Function Defects. *Haemophilia* **2008**, *14*, 1240–1249. [CrossRef] [PubMed]
3. Yeaman, M.R. Bacterial-Platelet Interactions: Virulence Meets Host Defense. *Future Microbiol.* **2010**, *5*, 471–506. [CrossRef]
4. Cox, D.; Kerrigan, S.W.; Watson, S.P. Platelets and the Innate Immune System: Mechanisms of Bacterial-Induced Platelet Activation. *J. Thromb. Haemost.* **2011**, *9*, 1097–1107. [CrossRef]
5. Hannachi, N.; Fournier, P.-E.; Martel, H.; Habib, G.; Camoin-Jau, L. Statins Potentiate the Antibacterial Effect of Platelets on *Staphylococcus aureus*. *Platelets* **2021**, *32*, 671–676. [CrossRef]
6. Hannachi, N.; Baudoin, J.-P.; Prasanth, A.; Habib, G.; Camoin-Jau, L. The Distinct Effects of Aspirin on Platelet Aggregation Induced by Infectious Bacteria. *Platelets* **2020**, *31*, 1028–1038. [CrossRef]
7. Hamzeh-Cognasse, H.; Damien, P.; Chabert, A.; Pozzetto, B.; Cognasse, F.; Garraud, O. Platelets and Infections—Complex Interactions with Bacteria. *Front. Immunol.* **2015**, *6*, 82. [CrossRef]
8. Ezzeroug Ezzraimi, A.; Hannachi, N.; Mariotti, A.; Rolain, J.-M.; Camoin-Jau, L. Platelets and *Escherichia coli*: A Complex Interaction. *Biomedicines* **2022**, *10*, 1636. [CrossRef]
9. Ezzeroug Ezzraimi, A.; Hannachi, N.; Mariotti, A.; Rolland, C.; Levasseur, A.; Baron, S.A.; Rolain, J.-M.; Camoin-Jau, L. The Antibacterial Effect of Platelets on *Escherichia coli* Strains. *Biomedicines* **2022**, *10*, 1533. [CrossRef]
10. Bessis, M. Studies in Electron Microscopy of Blood Cells. *Blood* **1950**, *5*, 1083–1098. [CrossRef]
11. Sullam, P.M.; Valone, F.H.; Mills, J. Mechanisms of Platelet Aggregation by Viridans Group Streptococci. *Infect. Immun.* **1987**, *55*, 1743–1750. [CrossRef] [PubMed]
12. White, J.G. Use of the Electron Microscope for Diagnosis of Platelet Disorders. *Semin. Thromb. Hemost.* **1998**, *24*, 163–168. [CrossRef] [PubMed]
13. White, J.G.; Krumwiede, M. Some Contributions of Electron Microscopy to Knowledge of Human Platelets. *Thromb. Haemost.* **2007**, *98*, 69–72. [PubMed]
14. Cattaneo, M.; Cerletti, C.; Harrison, P.; Hayward, C.P.M.; Kenny, D.; Nugent, D.; Nurden, P.; Rao, A.K.; Schmaier, A.H.; Watson, S.P.; et al. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. *J. Thromb. Haemost.* **2013**, *11*, 1183–1189. [CrossRef] [PubMed]
15. Dukes, M.J.; Ramachandra, R.; Baudoin, J.-P.; Gray Jerome, W.; de Jonge, N. Three-Dimensional Locations of Gold-Labeled Proteins in a Whole Mount Eukaryotic Cell Obtained with 3 nm Precision Using Aberration-Corrected Scanning Transmission Electron Microscopy. *J. Struct. Biol.* **2011**, *174*, 552–562. [CrossRef] [PubMed]
16. Reynolds, E.S. The Use of Lead Citrate at High Ph as an Electron-Opaque Stain in Electron Microscopy. *J. Cell Biol.* **1963**, *17*, 208–212. [CrossRef]
17. Watson, C.N.; Kerrigan, S.W.; Cox, D.; Henderson, I.R.; Watson, S.P.; Arman, M. Human Platelet Activation by *Escherichia coli*: Roles for FcγRIIA and Integrin AIIbβ3. *Platelets* **2016**, *27*, 535–540. [CrossRef]
18. Fejes, A.V.; Best, M.G.; van der Heijden, W.A.; Vancura, A.; Verschueren, H.; de Mast, Q.; Wurdinger, T.; Mannhalter, C. Impact of *Escherichia coli* K12 and O18:K1 on Human Platelets: Differential Effects on Platelet Activation, RNAs and Proteins. *Sci. Rep.* **2018**, *8*, 16145. [CrossRef]
19. Jorgenson, M.A.; Young, K.D. Interrupting Biosynthesis of O Antigen or the Lipopolysaccharide Core Produces Morphological Defects in *Escherichia coli* by Sequestering Undecaprenyl Phosphate. *J. Bacteriol.* **2016**, *198*, 3070–3079. [CrossRef]
20. Jeong, H.; Kim, H.J.; Lee, S.J. Complete Genome Sequence of *Escherichia coli* Strain BL21. *Genome Announc.* **2015**, *3*, e00134-15. [CrossRef]
21. Akashi, S.; Saitoh, S.; Wakabayashi, Y.; Kikuchi, T.; Takamura, N.; Nagai, Y.; Kusumoto, Y.; Fukase, K.; Kusumoto, S.; Adachi, Y.; et al. Lipopolysaccharide Interaction with Cell Surface Toll-like Receptor 4-MD-2: Higher Affinity than That with MD-2 or CD14. *J. Exp. Med.* **2003**, *198*, 1035–1042. [CrossRef]
22. Park, B.S.; Lee, J.-O. Recognition of Lipopolysaccharide Pattern by TLR4 Complexes. *Exp. Mol. Med.* **2013**, *45*, e66. [CrossRef]
23. Zhang, G.; Han, J.; Welch, E.J.; Ye, R.D.; Voyno-Yasenetskaya, T.A.; Malik, A.B.; Du, X.; Li, Z. Lipopolysaccharide Stimulates Platelet Secretion and Potentiates Platelet Aggregation via TLR4/MyD88 and the CGMP-Dependent Protein Kinase Pathway. *J. Immunol.* **2009**, *182*, 7997–8004. [CrossRef]
24. Matus, V.; Valenzuela, J.G.; Hidalgo, P.; Pozo, L.M.; Panes, O.; Wozniak, A.; Mezzano, D.; Pereira, J.; Sáez, C.G. Human Platelet Interaction with *E. coli* O111 Promotes Tissue-Factor-Dependent Procoagulant Activity, Involving Toll like Receptor 4. *PLoS ONE* **2017**, *12*, e0185431. [CrossRef]

Chapitre III : Étude de l'effet des antiplaquettaires sur l'interaction plaquettes-*E. coli*

Préambule

Le sepsis a été défini en 2016 comme étant « un dysfonctionnement d'un organe potentiellement mortel causé par une réponse dérégulée de l'hôte à l'infection » (51). En cas d'effraction vasculaire, les bactéries passent dans la circulation sanguine, ce qui génère des mécanismes de défense. L'activation de l'hémostase au niveau du site de la lésion et la formation de thrombi dans les capillaires locaux permettent non seulement de stopper l'hémorragie, mais aussi d'initier une réponse anti-infectieuse précoce. Ce dernier phénomène est un mécanisme de défense qui limite l'infection aux lésions par un processus appelé immunothrombose ou thrombo-inflammation (52). L'immunothrombose désigne la capacité des plaquettes à interagir, directement ou indirectement, avec les leucocytes pour induire une réponse immunitaire. Une fois activées, les plaquettes vont changer de conformation et libérer le contenu de leurs granules, qui contiennent un grand nombre de molécules, dont certaines sont impliquées dans la réponse inflammatoire. En cas de septicémie, ces réactions locales s'étendent à l'ensemble de l'organisme, produisant des phénomènes délétères dans de nombreux tissus. Cela conduit au développement d'un syndrome de défaillance multiviscérale (53).

Escherichia coli est une des bactéries les plus fréquemment impliquées dans le sepsis. Une méta-analyse de 2021 étudiant l'épidémiologie des agents responsables de bactériémies entre 2007 et 2018 dans les pays occidentaux estime qu'*E. coli* est retrouvé en moyenne dans 27.1% des cas de bactériémies, avec cependant une grande hétérogénéité en fonction des études, allant de 6.5 à 57% (54). La porte d'entrée principalement mise en évidence est le tractus uro-génital, responsable de plus de 50% des contaminations.

Un des modèles où la relation entre *E. coli* et plaquettes est bien décrite est celui SHU. Bien que les travaux réalisés sur les souches O111 et O157:H7 montrent que ces deux souches sont capables d'induire une agrégation plaquettaire, les récepteurs plaquettaires impliqués seraient différents. En effet, TLR-4 interagit avec la souche O:111 et FcγRIIA avec la souche O157:H7.

Les travaux de Watson *et al* et Fejes *et al* sont les principales études réalisées sur des souches de *E. coli* appartenant à d'autres pathovars (32,33). Dans ces travaux, l'agrégation plaquettaire observée est dépendante à la fois de la souche impliquée, ainsi que du ratio plaquettes/bactéries présent, démontrant que la concentration de l'inoculum joue aussi un rôle important. Le rôle clef du FcγRIIA et de l'intégrine αIIbβ3, est également démontré dans ces

modèles. Cette agrégation plaquettaire répond à la loi « du tout ou rien », ce qui signifie qu'une souche donnée sera capable d'induire une agrégation plaquettaire importante, ou de ne pas avoir d'effet.

Une des hypothèses qui permettrait de comprendre l'hétérogénéité de réponses des différentes souches repose sur la différence de structure des LPS. Certaines bactéries Gram-négatif possèdent un LPS-L, comme la souche *E. coli* O18 :K1, alors que d'autres présentent un LPS-R, comme la souche K12 d'*E. coli* (55). Les souches présentant un LPS-R activeraient un large spectre de cellules dont les macrophages, et avec une plus grande efficacité, que les souches avec un LPS-L. Ainsi, cette capacité ou non à faire agréger les plaquettes pourrait donc dépendre de la structure des LPS des bactéries Gram-négatif, et de leur capacité ou non à être reconnues par le TLR-4 exprimé à la surface des plaquettes. L'antigène-O pourrait également jouer un rôle important dans la reconnaissance de ces motifs bactériens par les plaquettes et dans l'induction ou non d'un choc septique.

Les voies de signalisation secondaires à l'interaction entre LPS et TLR-4 mettent en évidence une activation de MyD88, une activation de la voie PI3K/Akt, induisant une activation plaquettaire responsable ensuite de l'agrégation des plaquettes. Le LPS, en interagissant avec le TLR-4, n'entrainerait pas à lui seul une activation plaquettaire, mais jouerait un rôle facilitateur de l'adhésion plaquettaire, en association avec un agoniste plaquettaire, et favoriserait également la sécrétion et l'agglutination des plaquettes. Il existe une autre voie d'activation reposant sur la formation d'un complexe TLR-4-sCD14.

Ainsi, la réduction de l'activation plaquettaire par l'administration des AAP pourrait constituer une cible thérapeutique d'intérêt pour la prévention de la morbi-mortalité des patients atteints de sepsis (29). Les principales études ayant évalué le bénéfice potentiel des AAP au cours du sepsis sont présentées dans deux méta-analyses (29,56). Bien que les résultats soient en faveur d'un effet bénéfique des AAP, l'analyse des critères principaux, à savoir la morbi-mortalité, ne prennent pas en compte l'espèce bactérienne responsable de l'état septique. Il est donc difficile d'évaluer l'intérêt des AAP dans le cas spécifique des sepsis à *E. coli.*

En revanche, l'administration de LPS purifié chez des sujets sains prétraités par aspirine et anti-P_2Y_{12} induirait une diminution de la sécrétion de facteur de nécrose tumorale (TNF) par rapport à des sujets non pré-traités sans cependant diminuer la production d'autres cytokines pro-inflammatoires (57). Les plaquettes pourraient donc jouer un rôle de modulation de la réponse inflammatoire, en plus de leur effet bactéricide direct, qui pourrait être modifié par l'administration d'antiplaquettaires. Outre leurs actions classiques sur la voie du

TxA2 et du récepteur P_2Y_{12}, les antiplaquettaires pourraient avoir une action sur la voie de signalisation activée suite à l'interaction entre le TLR-4 plaquettaire et le CD14 soluble.

Dans le futur, l'identification de souches pro-agrégantes permettrait d'identifier les voies de signalisation impliquées dans l'activation plaquettaire, mais également de tester chacun des antiplaquettaires afin de déterminer quelle molécule permettrait d'agir de manière optimale afin de diminuer la réactivité plaquettaire lors du sepsis.

Article 4: Effect of antiplatelet agents on *Escherichia coli* sepsis mechanisms: a review

Mariotti, A.; **Ezzeroug Ezzraimi, A.;** Camoin-Jau, L.

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Effect of antiplatelet agents on Escherichia coli sepsis mechanisms: a review

Antoine Mariotti[1, 2, 3], **Amina Ezzeroug Ezzraimi**[2], **Laurence Camoin-Jau**[2, 1, 3*]

[1]Assistance Publique Hôpitaux de Marseille, France, [2]IHU Mediterranee Infection, France, [3]Faculté de Pharmacie, Aix Marseille Université, France

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Keywords

Escherichia coli, platelets, Platelet Aggregation, TLR4, Antiplatelet drugs, Sepsis

Abstract

Word count: 165

Despite ever-increasing improvements in the prognosis of sepsis, this condition remains a frequent cause of hospitalisation and mortality in Western countries. Sepsis exposes the patient to multiple complications, including thrombotic complications, due to the ability of circulating bacteria to activate platelets.

One of the bacteria most frequently implicated in sepsis, Escherichia coli, a Gram-negative bacillus, has been described as being capable of inducing platelet activation during sepsis. However, to date, the mechanisms involved in this activation have not been clearly established, due to their multiple characteristics. Many signalling pathways are thought to be involved

At the same time, reports on the use of antiplatelet agents in sepsis to reduce platelet activation have been published, with variable results. To date, their use in sepsis remains controversial.

The aim of this review is to summarise the currently available knowledge on the mechanisms of platelet activation secondary to Escherichia coli sepsis, as well as to provide an update on the effects of antiplatelet agents in these pathological circumstances.

Contribution to the field

Besides their well-known role in the mechanisms of haemostasis, platelets are involved in bacterial defence mechanisms. Very few works in the literature have been interested in the consequences of interactions between platelets and Gram-negative bacteria, in particular Escherichia coli. Frequently implicated in sepsis, Escherichia coli could be able to induce platelet activation during sepsis. However, to date, the mechanisms involved in this activation are not clearly established, due to their multiple characteristics. Many signalling pathways are thought to be involved. While antiplatelet agents were evaluated in sepsis, their use remains controversial. The aim of this review is therefore to summarize the currently available knowledge on the mechanisms of platelet activation secondary to Escherichia coli sepsis, as well as to provide an update on the effects of antiplatelet agents in these pathological circumstances. We consider that this review provides important data on the interactions between platelets and Escherichia coli.

Effect of antiplatelet agents on *Escherichia coli* sepsis mechanisms: a review

Antoine Mariotti[1,2,3], Amina Ezzeroug Ezzraimi[1,2], Laurence Camoin-Jau[1,2,3*]

[1]Aix Marseille Univ, IRD, APHM, MEPHI, IHU Méditerranée Infection, Marseille, France
[2] IHU Méditerranée Infection, boulevard Jean Moulin, 13385 Marseille, France
[3]Haematology Laboratory, Hôpital de la Timone, APHM, boulevard Jean- Moulin, 13005 Marseille, France

[*]**Corresponding author:**
Prof. Laurence Camoin-Jau
Aix Marseille University, IRD, MEPHI, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France.
Tel: 33 4 91 38 60 49
Fax: 33 4 91 38 91 55
E-mail: laurence.CAMOIN@ap-hm.fr

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Abbreviations

APA-: antiplatelet agent

ADP: Adenosine diphosphate

CCL5-: chemokine ligand 5

CXCL4-: platelet factor 4

DIC: disseminated intravascular coagulation

ClfA: clumping factor A

C4BP: C4 binding protein

EHEC: Enterohaemorrhagic *Escherichia coli*

EIEC: Enteroinvasive *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

ExPEC: Extraintestinal pathogenic *Escherichia coli*

FcγRIIA: immunoglobulin Fc fragment receptor IIa

TF: Tissue Factor

IgG: immunoglobulin G

LPS: Lipopolysaccharide

MODS: Multiple organ dysfunction syndrome

NET: Neutrophil extracellular traps

NO: Nitrogen monoxide

PAF: Platelet Activating Factor

PAF-R: PAF Receptor

PI3K : Phosphoinositide 3-kinase

PSGL-1 : P-selectin glycoprotein ligand-1

RR: Relative Risk

sCD14: soluble CD14

ARDS: Acute Respiratory Distress Syndrome

HUS: Haemolytic Uremic Syndrome

SIRS: Systemic Inflammatory Response Syndrome

TLR-4: Toll-like receptor 4

TxA2: Thromboxane A_2

von Willebrand factor: vWF

Summary

Despite ever-increasing improvements in the prognosis of sepsis, this condition remains a frequent cause of hospitalisation and mortality in Western countries. Sepsis exposes the patient to multiple complications, including thrombotic complications, due to the ability of circulating bacteria to activate platelets.

One of the bacteria most frequently implicated in sepsis, *Escherichia coli*, a Gram-negative bacillus, has been described as being capable of inducing platelet activation during sepsis. However, to date, the mechanisms involved in this activation have not been clearly established, due to their multiple characteristics. Many signalling pathways are thought to be involved

At the same time, reports on the use of antiplatelet agents in sepsis to reduce platelet activation have been published, with variable results. To date, their use in sepsis remains controversial.

The aim of this review is to summarise the currently available knowledge on the mechanisms of platelet activation secondary to *Escherichia coli* sepsis, as well as to provide an update on the effects of antiplatelet agents in these pathological circumstances.

Introduction

In addition to their role in haemostasis, platelets play a major role in the anti-infective response and in the regulation of the inflammatory response (1). This anti-infective defence role of platelets has been demonstrated by their ability to interact and activate in the presence of many classes of pathogens. They are involved in antiviral defence, notably through the release of the chemokine CCL5, promoting the development of a protective response during dengue virus (2,3) and hepatitis C virus (HCV) infection (4). More recently, platelets have also been described to be involved in a deleterious response during SARS-CoV-2 infection, linked to abnormal expression of certain genes (5), making platelets hyper-reactive and promoting the procoagulant state found in critical patients with COVID-19 (6,7).

Platelet activation may also play an important role in the pathophysiological mechanisms of certain parasitic infections, such as malaria, where platelets are a key player in the neurological complications of malaria due to their ability to form microthrombi. This response initially limits parasite proliferation and has a protective effect on the host, but will later become deleterious if platelet activation persists (8).

Many receptors located on the surface of platelets have been shown to be involved in the interaction with bacteria, such as TLRs, the PAF receptor, FcγRIIA or GPIbα (9). During sepsis, bacteria will be able to interact with one or more of these receptors and induce platelet activation that can lead to the appearance of deleterious phenomena, such as the appearance of thrombosis or deregulated inflammation, or beneficial, with a demonstrated bactericidal effect of platelets on certain bacterial strains (10).

Sepsis is characterised by complex pathological mechanisms and is associated with a high mortality rate (11). In 1991, a consensus conference proposed the initial definition of sepsis as “a syndrome of systemic inflammatory response (SIRS) of the host to an infection” (12). In 2016, a new definition of sepsis was developed as “life-threatening organ dysfunction caused by a dysregulated host response to infection”. (13). Sepsis is an extremely serious condition in which bacteria induce the activation of haemostasis and, in particular, the activation of platelets across the entire vascular system, leading to the phenomena of immuno-thrombosis (14), which is based on an

uncontrolled interaction of the systems of inflammation and haemostasis, with platelets being an integral part of both systems.

In this review, we will focus on *Escherichia coli* sepsis. We will review the current state of knowledge on the mechanisms of interaction between platelets and *Escherichia coli*, and the potential value of antiplatelets in this indication.

Platelets in the pathophysiology of sepsis

Platelet-bacteria interactions during sepsis

In recent years, a growing number of studies have demonstrated that platelets are involved in the deleterious processes observed during sepsis and that they play an important role in the development of organ damage that can lead to multiple organ dysfunction syndrome (MODS) (15).

In the event of vascular invasion, bacteria enter the bloodstream, which triggers defence mechanisms. Activation of haemostasis at the site of the injury and the formation of thrombi in local capillaries not only stops bleeding, but also initiates an early anti-infective response. Platelets will express receptors (P-selectin, CD40L or CD154) allowing interaction with immune cells or with endothelial cells (via PSGL-1, CD154 receptors) which allow signal transduction and activation of these different cell types (16). This phenomenon is a defence mechanism that limits infection of the lesions by a process known as immunothrombosis or thromboinflammation (14). In the case of sepsis, local reactions extend to the whole body, producing deleterious phenomena in many tissues. Thus, in an animal model of abdominal sepsis, neutrophil infiltration of the lung, induced by platelet activation, is thought to contribute towards the development of pulmonary oedema (17).

At the same time, endothelial activation, observed during sepsis, leads to the appearance of or increase in surface molecules, such as von Willebrand factor **(vWF)**, E-selectin and integrins $\alpha_V\beta_3$, encouraging interaction with platelets and leading to their activation, while decreasing anti-adhesive inhibition pathways, thus favouring the risk of thrombosis (18,19) (Figure 1). Ischaemia in several areas, secondary to the

appearance of generalised activation of endothelial cells, may be observed, leading in particular to abnormalities in blood pressure and vascular permeability (20) associated with the formation of micro clots.

Several types of signalling mechanisms and pathways may be involved depending on the bacterial species, or on the receptor implicated in this interaction.

Platelets and immune cells

Platelets can therefore exhibit several effects that will take place during sepsis in a chronological manner. Firstly, they will be involved in the recognition of specific bacterial patterns, notably through TLRs (21,22). Once the bacteria have been recognised, a platelet response will occur, which will vary according to several parameters: the bacterial species involved and their escape mechanisms, and the platelet receptors and signalling pathways involved. Platelets can therefore be involved in the destruction of pathogens, either by direct cytotoxicity (10,23) or by cooperation with other cell types, through opsonisation (24) or NETosis (25,26). By activating, platelets will also cause the release of chemical mediators (CCL5, CXCL4), which will be involved in chemotaxis of certain immune cells, notably neutrophils and monocytes (27,28), but also in the modulation of the inflammatory response (29). This interaction with the inflammatory system will take place via certain cytokines (30) or via the complement system (31,32). Platelets will also be able to interact with the coagulation system and remain a major player in the initiation of DIC and the procoagulant state encountered during sepsis (33,34). In fact, recent studies show that there are platelet subpopulations, including pro-coagulant platelets that arise in response to intraplatelet calcium release. These pro-coagulant platelets will play a key role in the regulation of thrombotic and haemorrhagic phenomena, but also in thromboinflammation and NETosis, by interacting with certain immune cells, notably neutrophils (35).

The deregulation of the NETosis phenomenon, encountered during sepsis, may lead to the development of deleterious effects, relying on unregulated activation of neutrophils in response to a platelet-derived signal to scavenge circulating bacteria (25). However, the formation of these NETs will also lead to the formation of a pro-

coagulant terrain allowing the attachment of certain coagulation factors or extracellular vesicles (36), which can favour the development of arterial and venous thrombotic phenomena (37). These deleterious effects, when prolonged, can lead to either localised organ dysfunction or to multiple organ failure syndromes. The organs most frequently concerned are the kidneys, through the development of acute renal failure secondary to renal hypoperfusion, but also secondary to endothelial damage (38); the liver, which is the site of the synthesis of numerous cytokines, and which can therefore play an important role in the anarchic inflammation that occurs during sepsis (39); and the circulatory system, notably through the systemic activation of endothelial cells and the significant release of vasodilator molecules such as nitric oxide (NO), leading to hypotension that is almost always encountered during sepsis (40).

Some bacteria also appear to have escape mechanisms from the platelet-induced anti-infective response. *Yersinia pestis*, for example, is able to induce a change in the structure of the thrombus, formed by platelets and fibrin, in order to escape the NETosis phenomenon. This is possible through one of its virulence factors (Y pestis plasminogen activator Pla) which activates a fibrinolysis phenomenon, allowing an escape from bactericidal action (41).

Other actors of thrombosis in sepsis

However, platelets are not the only factors linking haemostasis and inflammation. Certain mediators of inflammation have the ability to interact with different factors in the coagulation cascade, creating a pro-thrombotic state. The coagulation and complement systems, usually represented separately, are in fact closely intertwined. Proteins involved in one of the cascades are capable of interacting with factors in the other system. For example, the activation of factor XII to activated factor XIIa is capable of inducing the activation of the classical complement pathway, while C4-binding protein (C4BP) can bind to protein S and inhibit its effect, thereby promoting the development of thrombosis (42). The existence of exacerbated inflammation can therefore lead to the development of hypercoagulability and, in the most severe cases, induce the onset of disseminated intravascular coagulation (DIC),

thus increasing the risk of developing tissue hypoxia and organ dysfunction through the formation of thrombi in the capillary circulation (43,44).

In summary, this inflammation-coagulation phenomenon in sepsis, associated with endothelial damage, is partly the result of the activation of platelets, which are able, through some of their membrane receptors, to participate in anti-infective defence. Platelets, once activated, will exacerbate systemic inflammatory reactions and coagulation disorders through interactions with immune cells and endothelial cells. The platelet activation observed during sepsis could also partly explain the thrombocytopaenia frequently observed during sepsis, through a consumption mechanism (45). In addition to the already high mortality rate in sepsis, linked to the intrinsic severity of the disease, the occurrence of thrombocytopaenia further worsens the prognosis, exposing the patient to higher morbimortality (46). This includes an increased risk of bleeding, the development of acute renal failure, a longer stay in intensive care, and even mortality if the thrombocytopaenia is not resolved (47).

Thus, the inhibition of platelet activation may reduce uncontrolled inflammatory and coagulation reactions in sepsis, thereby reducing the severity of organ damage and improving patient prognosis (48).

Escherichia coli sepsis

A 2021 meta-analysis studying the epidemiology of bacteraemia-causing agents between 2007 and 2018 in Western countries estimates that *Escherichia coli* is found in an average of 27.1% of bacteraemia cases, although there is considerable heterogeneity between studies, ranging from 6.5% to 57% (49). The main entry point identified was the urogenital tract, responsible for more than 50% of infections. According to this meta-analysis, the overall incidence of *Escherichia coli* bacteraemia, all groups combined, is 40.2 to 57.2 per 100,000 inhabitants per year, with a mortality rate of between 2.9 and 10.3 per 100,000 people. On a smaller scale, a 2019 UK report indicated that the incidence rate of *Escherichia coli* bacteraemia has been increasing over the past 10 years, with a significant acceleration since 2014, from 55.2 per 100,000 population in 2014 to 70.7 cases per 100,000 population (50).

However, these figures remain global statistics, and should be put into perspective according to the sex and age of the patients as well as their underlying pathologies. The incidence is higher in women than in men, and increases sharply with age, with statistically higher rates in the general population from the age of 60. Indeed, the incidence is multiplied by 30 in subjects over 75 years of age compared to young adults (24). The subgroup analysis in this meta-analysis shows that patients with haematological malignancies are most at risk of developing *Escherichia coli* sepsis. A study in a Swedish centre found a prevalence of up to 12.7% in patients with chronic lymphocytic leukaemia (51). Among other haematological diseases, *Escherichia coli* is responsible for 46% of bacteraemias in acute leukaemia (52) and 22.4% in multiple myeloma (53). In addition, *Escherichia coli* is found in 34.2% of neutropenic patients with sepsis (54). *Escherichia coli* is also found in patients with solid cancers. In a 2014 study, it was implicated in 30.5% of bacteraemias (55) and more precisely in 22.2% of patients with colon cancer (56). Finally, surgical patients are also at risk of developing *Escherichia coli* sepsis, particularly those who have undergone abdominal surgery, due to the important localisation of this pathogen in the digestive tract. *Escherichia coli* was implicated in more than a quarter of cases of sepsis after pancreatic resection and in 12.4% of gastric resections (57). These figures are consistent with those of another retrospective study on the development of septic shock after digestive surgery, where *Escherichia coli* was found in 16.8% of cases (58).

However, cancer is only the third most common relative risk for developing *Escherichia coli* bacteraemia (RR: 14.9). Indeed, this relative risk is 26.9 for patients with renal failure on dialysis and 20.for patients with solid organ transplantation (49).

Escherichia coli sepsis also affects other categories of patients. A retrospective study carried out in Ireland showed that between 2001 and 2014, *Escherichia coli* was involved in 37% of cases of sepsis in pregnant women, a population particularly at risk, as sepsis accounts for a quarter of maternal deaths in pregnancy (59). Similarly, neonates are a particularly high-risk patient group. Although Streptococcus B (*Streptococcus agalactiae*) is the most frequently implicated germ in newborn sepsis (38%–43% of cases), *Escherichia coli* sepsis is the second most important cause of mortality (24% of all episodes), being implicated in 24.5% of sepsis-related deaths

(60). This high mortality is partly explained by the fact that 81% of *Escherichia coli* bacteraemias occur in premature infants, who are at greater risk of infection due to their as yet fragile immunity (61).

Escherichia coli: general information, classification and pathogenicity

Escherichia coli is a Gram-negative commensal bacterium belonging to the Enterobacteriaceae family, frequently found in the human digestive tract and representing a large part of the intestinal flora. Certain strains are often found in human pathologies, particularly in community and nosocomial infections, in a wide variety of sites, including meningitis, gastroenteritis, and urinary tract infections (62). These *Escherichia coli* strains are capable of acquiring virulence factors (adhesins, capsule, synthesis and secretion of toxins, etc.) which confer their pathogenic power, as well as antibiotic resistance mechanisms which give them reduced sensitivity to certain anti-infective molecules (63,64).

In 2016, a classification of *Escherichia coli* strains into several subclasses was proposed (65) according to the syndromes they are capable of causing, which themselves depend on the different virulence factors that the strain may have acquired. A distinction must be made between enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteropathogenic (EPEC) and enteroinvasive (EIEC) strains, which are similar to Shigella. These pathogenic strains all have a tropism for the digestive tract and will cause gastrointestinal manifestations. Commensal strains, on the other hand, are only rarely pathogenic, in cases of extra-intestinal dissemination linked to particular circumstances (major immunodepression, abdominal trauma, etc.). Finally, it is important to distinguish strains that cause extra-intestinal pathogens (ExPEC), which can reach many organs, but which are often found as commensals of the digestive tract. These strains have a particular ability to disseminate and survive in a normally sterile site, leading to colonisation and potential infection in these extra-intestinal locations.

However, although *Escherichia coli* is frequently encountered in clinical practice, very few studies have investigated the mechanisms of *Escherichia coli*-

platelet interactions. Despite a growing interest in Gram-negative bacilli, including *Escherichia coli*, studies on bacterial interactions leading to platelet activation have been mainly limited to Gram-positive bacteria, including staphylococci (*Staphylococcus aureus* and *Staphylococcus epidermidis* (66,67)) and streptococci (*Streptococcus sanguinis* and *Streptococcus gordonii* (68,69)).

Platelet-*Escherichia coli* interactions

Three mechanisms could explain the interaction between bacteria and platelets (9,70) responsible for platelet activation. **Bacteria can bind to platelets via a plasma protein. This is the case for *Staphylococcus aureus* and *Helicobacter pilori*, which are able to bind to vWF. Bacteria can direct binding to a platelet receptor. *Streptococcus gordonii* and *Streptococcus sanguinis* can directly interact with GpIb via their Hs antigen. This interaction can also be mediated by secreted bacterial proteins, i.e., toxins.**

When activated, platelets secrete the contents of their granules, which contain more than 300 molecules (71) including adenosine diphosphate (ADP) and serotonin. Secreted cytokines and chemokines recruit leukocytes, and secreted antimicrobial peptides act to kill pathogens. This ability to activate in response to infection thus gives them the ability to destroy bacteria through bactericidal activity (10). This demonstrates that their activation and degranulation play an important role in the fight against infection.

The presence of multiple mechanisms makes it difficult to identify the roles of different proteins (both bacterial and platelet). This analysis is further complicated by the fact that interactions are not only species-specific but also strain-specific, as demonstrated in 2016 by Watson *et al.* (10,72,73). Some interactions lead to platelet activation, while others will have no direct effect. One study even highlights the fact that LPS promotes a dose-dependent decrease in platelet reactivity in response to certain agonists, notably thrombin or ADP. This inhibition of platelet aggregation would be due to modifications in the concentration of numerous substances, such as thromboxane A_2 or cyclic GMP (74).

Non-activating interactions are generally of high affinity and probably play a role in supporting platelet adhesion under the shear conditions encountered in the circulation (75). Typically, the bacterial proteins involved in adhesion are distinct from those that induce aggregation. Thus, bacteria can promote platelet adhesion and/or trigger platelet activation. Platelet activation is characterised by the appearance of or increase in certain platelet surface markers, or by the secretion of granular content (CD42b, P-selectin (CD62P) and activated GpIIbIIIa (CD41)). These markers are most often detected by flow cytometry, but do not necessarily indicate the formation of a platelet aggregate (76).

One model in which the relationship between *Escherichia coli* and platelets has been well described is haemolytic uremic syndrome (HUS), which is characterised by, among other things, mechanically induced haemolytic anaemia and thrombocytopenia due to activation of platelets by altering the vascular endothelium caused by the production of Shigatoxins (77). Work on the *Escherichia coli* O111 strain has led to a better understanding of these interactions. This strain of *Escherichia coli* O111 producing these Shigatoxins could interact directly with platelets via Toll-like Receptor 4 (TLR4), leading to an increase in platelet activation markers and the expression of tissue factor (78). The complement system, as well as variations in the LPS O antigen, could explain the observed platelet activation (79). However, these studies did not establish the predominant interaction mechanism for all *Escherichia coli* strains, which appears to be strain-dependent. Indeed, the *Escherichia coli* O157:H7 strain, which is also responsible for HUS, is thought to induce FcγRIIA receptor-mediated platelet aggregation rather than TLR4 (80).

Although as early as 1971, a paper demonstrated the ability of *Escherichia coli* to induce platelet aggregation (81), it was not until several decades later that the mechanisms of this platelet aggregation were studied and that some interest was shown in other strains of *Escherichia coli*. The study by Watson *et al*. identified two important characteristics. The platelet aggregation they are able to induce is dependent on both the strain involved, as well as the platelet/bacteria ratio, demonstrating that the concentration of the inoculum also plays an important role. This platelet aggregation responds to the "all or nothing" law, which means that a given strain will either be able

to induce significant platelet aggregation or will have no effect. In this study, the key role of FcγRIIA and integrin αIIbβ3, also known as GpIIbIIIa (72) is also described, although, as we have seen, these signalling pathways may play a different role depending on the strains involved. This notion was confirmed by Fejes *et al*. on the reference strain K12, which is reported to induce an elevation of the platelet activation markers P-selectin, CD63, the GPIIbIIIa activation marker PAC-1, and bound fibrinogen (82). In the paper by Fejes *et al.*, *Escherichia coli* strains were classified according to the structure of their lipopolysaccharide (LPS), specifically lipid A, which is thought to partly determine their interaction with platelets as well as with certain immune system cells. Some Gram-negative bacteria have a 'smooth' LPS, such as *Escherichia coli* O18:K1, while others have a 'rough' LPS, such as *Escherichia coli* K12 strains (83). Strains with a 'rough' LPS would activate a wider spectrum of cells including macrophages, and with greater efficiency, than strains with a 'smooth' LPS (82). Thus, this ability or inability to aggregate platelets could depend on the structure of the LPS of the Gram-negative bacteria, and their ability or inability to be recognised by the TLR4 expressed on the surface of platelets. The O antigen may also play an important role in the recognition of these bacterial patterns by platelets and in the induction or non-induction of septic shock (79). The serotype of the strain involved in an infection in human pathology could thus play an important role in the prognosis of the patient.

The demonstration of TLR expression, particularly TLR4, on the surface of platelets has confirmed the hypothesis of a role for platelets in the anti-infective response (84). The interaction between LPS from Gram-negative bacteria and platelets will lead to an increase in platelet binding to fibrinogen in a possibly TLR4-dependent manner (82). Another major consequence of the presence of these TLRs on the platelet surface is the ability of platelets to be sequestered in the lung through their interaction with neutrophils (22).

The signalling pathways secondary to the interaction between LPS and TLR4 have been extensively studied to determine whether these receptors were indeed responsible for the platelet activation observed upon contact with *Escherichia coli*. The various platelet TLRs were able to induce, via activation of MyD88 (85,86) leading to

activation of the PI3K/Akt pathway, leading to platelet activation and subsequent platelet aggregation. LPS, by interacting with TLR4, would not by itself lead to platelet activation, but would play a facilitating role in platelet adhesion, in association with a platelet agonist, and would also promote platelet secretion and aggregation (86,87). It thus appears that *Escherichia coli* LPS, depending on the strain used, has a variable capacity to induce platelet aggregation. Similarly, in addition to its effect on platelet TLR4, the ability of *Escherichia coli* LPS to interact with TLR4 of other cell types could induce the production of extracellular microvesicles with a strong procoagulant potential, which may partly explain the risk of DIC in infected patients (88). In addition to being involved in the synthesis of microvesicles, platelets are capable of internalising their own microvesicles. This ability of platelets to endocytose microvesicles is thought to be dependent on the TLR4 receptor, and to promote the development of a prothrombotic state (89). However, platelets are not the only actors involved in the release of procoagulant microvesicles. The role of monocyte-derived microvesicles, containing significant amounts of tissue factor, in the development of thrombotic complications of sepsis has also been demonstrated (90,91).

TLR4 would not recognise LPS from different strains in the same way. Some forms would be able to bind only the TLR4 receptor, while others would induce the formation of a TLR4-sCD14 complex, which would involve specific signalling pathways (92). Since platelets do not produce sCD14, it would be adsorbed onto the platelet surface from the plasma (93). This effect would be very specific to activation by LPS and would not be observed with conventional agonists such as TRAP. CD14 plays a critical role in the physiopathology of sepsis. Its inhibition would attenuate the deleterious responses linked to pro-inflammatory cytokines and reduce the procoagulant state that accompanies sepsis (94,95).

Knowledge of this second activation pathway by the TLR4-sCD14 complex is particularly important, since it would lead to the release of CD40L contained in platelet granules (93) which would be able to induce or promote platelet aggregation, by having some affinity for integrin αIIbβ3 (96) integrin or by raising thromboxane A_2 levels (97). LPS is thought to cross-react with the PAF receptor, PAF-R. PAF is an

agent capable of causing intense platelet activation and disseminated intravascular coagulation syndromes very rapidly after injection in mice, leading to the death of the animal (98). The ability of LPS to induce activation of the PAF-R receptor has been known for many years. Indeed, LPS increases the expression of PAF-R *in vitro*, while potentiating its effect, even if the effects *in vivo* were more moderate (99). Furthermore, LPS, in addition to PAF-R-mediated platelet aggregation, is capable of inducing a tolerance phenomenon and thus of decreasing the response of platelets to PAF in the event of prior exposure, as well as modifying the expression of certain genes coding for pro-inflammatory cytokines (100). These different properties of LPS thus show that bacteria, through their structural proteins, are able to induce a complex platelet response mediated by numerous signalling pathways. This effect described on PAF is particularly interesting. Indeed, although the percentage of platelet aggregation measured by aggregometry decreased in response to antiplatelet drugs, there was still an ability of platelets to aggregate in the presence of PAF. This residual aggregation is not observed when platelets are activated by ADP (101).

FcγRIIA may also play a role in platelet activation. The anti-infective role of platelets is thought to be partly dependent on this receptor, their bactericidal activity being linked to the recognition of IgG deposited on bacteria during opsonisation (101). Therefore, these IgGs, via the Fc fragment, will be recognised by FcγRIIA and induce a platelet response capable of killing these bacteria. This FcγRIIA-dependent pathway is also involved in the platelet aggregation mechanism when platelets are exposed to bacteria, through the formation of immune complexes (102). Contact of a strain of *Escherichia coli* responsible for HUS (O157:H7) with platelets would trigger strong platelet aggregation, which would be completely inhibited in the presence of an anti-FcγRIIA (80). In contrast, the aggregation induced by this strain of *Escherichia coli* is not dependent on the TLR4 signalling pathway. The multiplicity of platelet activation mechanisms depending on the *Escherichia coli* strain testifies to the complexity of the interactions. Indeed, the mechanism involved seems to vary according to the virulence factors possessed by the strain. In the study by Watson *et al.,* activation of FcγRIIA was in close collaboration with integrin-dependent αIIbβ3 signalling involved in

platelet aggregation induced by some Gram-positive bacteria, notably Staphylococci (9).

Other phenomena have also been highlighted to explain the platelet activation occurring during sepsis, which would in fact be multifactorial and not solely dependent on direct activation by bacteria. The appearance of endothelial cell lesions during sepsis would favour not only inflammatory but also thrombotic phenomena (20,103). These endothelial lesions can lead to the appearance of platelet activation signals, which contribute to the phenomenon of aggregation *in vivo*. Indeed, the endothelium will interact with certain bacterial structures, leading to its activation, the release of numerous molecules (pro-inflammation cytokines, chemokines, pro-coagulant factors) as well as the expression of adhesion molecules (VCAM-1, PECAM), favouring interactions with the figurative elements of the blood, including platelets (104). The increase in these interactions, associated with an imbalance in the anticoagulant and antiaggregant function of the endothelium, would therefore increase the risk of thrombosis (105).

The anti-infective activity of platelets gives them a bactericidal power, which can be based on two mechanisms: indirect interaction with immune cells, or direct secretion by platelets of peptides with antimicrobial activity. These molecules belong to the platelet microbicidal peptide (PMP) family (106,107), which includes CXCL4, CXCL7 (also known as PBP), and CCL5, but also the class of defensins (human β-defensin 2 (BD2)); thymosin β4 (Tβ4)) and derivative antimicrobial peptides (thrombocydins, fibrinopeptide A) (108–110). In a recent study, we demonstrated that the bactericidal activity of platelets was heterogeneous and depended on the *Escherichia coli* strain involved: out of ten strains tested, only three induced bactericidal activity from platelets. Comparison of the genomes of two strains with different behaviours revealed the existence of differences in the cluster of genes involved in O antigen synthesis.

Based on our results and the literature, we hypothesised that the structural variations of LPS could alter the interactions with platelets and lead to a loss of the ability of platelets to activate and induce a bactericidal response. The loss of this bactericidal mechanism induced by platelets secondary to a modification of LPS

would thus be similar to what can be observed during resistance to certain antibiotics and would rely on the same mechanisms (111). However, few studies have looked at whether or not there is a link between antibiotic resistance and the ability of strains to interact with platelets. In our recent publication, we have shown that there appeared to be no link between colistin resistance and the ability of platelets to induce bacterial growth reduction using ten *Escherichia coli* strains (10).

There will, therefore, be a balance between the beneficial and deleterious effects of platelets during sepsis (112). They will have a direct antimicrobial effect, tissue repair capabilities and will allow immunomodulation of the immune response as well as some chemotaxis. However, uncontrolled and disseminated activation can lead to the aggravation of sepsis, through the thrombotic and haemorrhagic risk that DIC can induce. The use of molecules capable of reducing this state of platelet hyperactivation could therefore have a beneficial effect on mortality during sepsis.

Effect of antiplatelets in sepsis

Platelet count is included in the SOFA score and is inversely associated with the severity of sepsis (113). The severity of thrombocytopaenia is often used to stratify patients with sepsis and septic shock. In general, 20%–58% of septic patients develop thrombocytopaenia, of which 10% develop severe thrombocytopaenia (114).

Many mechanisms have been proposed to explain thrombocytopaenia in sepsis. A combination of several mechanisms remains the most likely hypothesis. Among other things, immune-mediated platelet activation decreases platelet life span, as activated platelets are rapidly cleared from the circulation (115). Thus, reducing platelet activation could be a therapeutic target of interest for the prevention of morbidity and mortality in affected patients (112). One fairly obvious hypothesis would, therefore, seem to be that, by reducing platelet reactivity, it would be possible to reduce their interactions with pathogens and the resulting consequences. Antiplatelets could, therefore, theoretically play an interesting role in improving the clinical prognosis during sepsis.

559 Several antiplatelet agents (APAs) are available that have a specific action on
560 one of the platelet activation pathways. The most commonly used APAs are aspirin,
561 which inhibits the synthesis of thromboxane A_2 (TxA_2), and inhibitors of the P_2Y_{12}
562 pathway (clopidogrel, prasugrel, ticagrelor). Other antiplatelet agents, notably the anti-
563 GpIIbIIIa, specifically inhibit platelet aggregation, have more limited indications and
564 have been little evaluated, to our knowledge, in this indication.
565 Although many studies have evaluated the potential benefit of APAs in sepsis, the data
566 of interest were presented in two meta-analyses and one literature review that aimed to
567 determine whether APA administration had a beneficial effect on reducing the risk of
568 mortality in sepsis (79, 84-85). The first meta-analysis included 15 studies conducted
569 between 2011 and 2016, and concluded that there was a reduction in the risk of
570 mortality of an average of 7%, ranging from 2% to 12% when aspirin was taken before
571 the development of sepsis (116). The analysis by Ouyang *et al.,* published in 2019,
572 includes 10 studies, of which four were also analysed by Trauer *et al.* (117).
573 In both meta-analyses, the authors point to significant heterogeneity between
574 studies. Indeed, although the results presented for each study were those of the subset
575 of patients with sepsis, some of the studies included in these meta-analyses not only
576 looked at cohorts of patients with sepsis, but also examined the effect of aspirin on the
577 development of acute respiratory distress syndrome (ARDS) (118,119) or acute
578 community-acquired pneumonia (120,121). Similarly, some studies looked at the
579 effect of long-term aspirin use on the development of the acute episode, while others
580 assessed its effect on mortality during hospitalisation. However, the benefit of the
581 administration of APAs and aspirin in particular is retained by these two meta-
582 analyses.
583 A review of the literature published in 2018 by Wang *et al.* (122) includes eight
584 retrospective studies conducted between 2012 and 2016. Some of these conclude that
585 there is a reduction in mortality when aspirin is taken in the ICU (120,123), while
586 others do not show statistically significant benefits (124). Conversely, only one study
587 showed an increased risk of developing severe sepsis in the ICU for patients who are
588 given aspirin (125).

589 The two meta-analyses (116,117) would therefore be in favour of a beneficial effect of
590 aspirin in terms of mortality, whereas the review of the literature by Wang *et al.* is
591 more reserved as to the conclusions of the effect of aspirin (122).
592 It is important to note that these clinical studies on the effect of APAs do not
593 assess their potential benefit in relation to the bacterial species responsible for the
594 septic condition. Some studies that have looked specifically at the Staphylococcus
595 family have shown that aspirin and ticagrelor have a greater effect on *Staphylococcus*
596 *sanguinis* than on *Staphylococcus aureus* (126). The effect of antiplatelet agents
597 therefore appears to be variable depending on the bacterial species.
598 The retrospective study by Osthoff *et al*. is unique in that it only looked at the
599 effect of aspirin in sepsis caused by *Staphylococcus aureus* in hospitalised patients
600 with or without long-term low-dose aspirin therapy (127). Interestingly, the control
601 population consisted of patients with *Escherichia coli* sepsis. The results indicated a
602 significant reduction in mortality with aspirin in the *Staphylococcus aureus* sepsis
603 group. In the *Escherichia coli* sepsis group, no reduction in mortality was observed
604 with aspirin use. However, they did not necessarily attribute this result to treatment
605 failure but to a much lower mortality rate than in the *Staphylococcus aureus* group,
606 with a lack of statistical power to assess this parameter (127). In view of these data, it
607 is difficult to confirm a possible beneficial role for aspirin in *Escherichia coli* sepsis.
608 However, a 2017 randomised study looked at the effect of antiplatelets when
609 healthy subjects were given purified LPS from *Escherichia coli* O:113 (128). Taking
610 aspirin for seven days before the administration of LPS would lead to an increase in
611 the inflammatory response, by increasing the production of pro-inflammatory
612 cytokines without, however, affecting the production of anti-inflammatory cytokines.
613 However, dual therapy, with the addition of a P_2Y_{12} inhibitor, reduces TNF-α
614 production to levels comparable to those observed with placebo without, however,
615 reducing the production of other pro-inflammatory cytokines. Platelets could therefore
616 play a role in modulating the inflammatory response, in addition to their direct
617 bactericidal effect, which could be modified by the administration of antiplatelets, and
618 in particular aspirin. One of the reasons for the variability of the platelet response upon
619 contact with different bacterial strains may be the affinity of the binding to TLR4 and

620 the formation of the TLR4-sCD14 complex. Antiplatelets will also play an important
621 role in this signalling pathway. sCD40L, released from platelet granules, is able to
622 induce an increase in the secretion of thromboxane A_2, and to potentiate the capacity
623 of platelets to aggregate. Aspirin intake would inhibit this thromboxane A_2 secretion
624 without affecting CD40L levels, thereby fully or partially suppressing the potentiating
625 effect of platelet aggregation in response to low doses of thrombin or collagen (97).
626 Aspirin is also thought to inhibit the phosphorylation of Myosin Light Chain (MLC), a
627 protein involved in modifying the actin cytoskeleton structure of platelets, allowing
628 them to remain in a resting conformation. CD40L levels in patients treated with
629 different antiplatelet agents, shows that CD40L levels are indeed not significantly
630 altered when taking aspirin, but that these levels are lowered when taking a P_2Y_{12}
631 inhibitor (129). A decrease in the membrane expression levels of P-selectin and
632 GpIIbIIIa was also observed (130).
633 In sepsis, CD40L has been used as a possible marker of platelet activation being
634 statistically higher in a group of patients admitted to intensive care compared to a
635 control group (131). These results confirm that sCD40L could be an important
636 prognostic marker in sepsis, with not only increased levels in septic patients, but also a
637 significant association with mortality (132).
638
639 Although the vast majority of studies on the effect of antiplatelets in sepsis have
640 focused on the effect of aspirin and P2Y12 ADP receptor inhibitors, other molecules
641 with a different mechanism of action could play an interesting role. This is particularly
642 the case with inhibitors of the PAR-1 (Protease-activated Receptors) to thrombin, such
643 as vorapaxar and atopaxar, which are still in development (133), and which would
644 therefore make it possible to obtain a reduction in thrombotic events secondary to
645 thrombin-related platelet activation, without any consequence on normal haemostasis.
646 However, although there are few data on the effect of these two molecules during
647 sepsis, it was shown in a randomised double-blind trial that vorapaxar was able to
648 induce a decrease in certain coagulation markers, notably the concentration of
649 prothrombin F1+2 fragments and thrombin-antithrombin complexes (TAT) after
650 injection of LPS into healthy subjects (134). This molecule was also capable of

inducing a decrease in the levels of antigenic Willebrand factor (vWF: Ag) and certain pro-inflammatory cytokines, notably TNF- and IL-6. Vorapaxar would therefore have a beneficial effect during sepsis, not only by reducing platelet activation, but also by its ability to act on other cell types. Monocytes play a fundamental role in the activation of coagulation during sepsis, notably through the significant release of tissue factor (TF), which is thought to be secondary to the activation of PAR-1 receptors on their surface (135,136). If we look more specifically at the effect of these PAR-1 inhibitors during an Escherichia coli infection, we find a study that was particularly interested in the effect of one of these molecules, SCH79797, which not only induces an intense formation of NETs, but also has a direct antibiotic effect against the outer membrane of *Escherichia coli* (115). However, these effects would not be found with vorapaxar, and would therefore not be dependent on PAR-1 inhibition.

Conclusion

The interaction between platelets and bacteria is a complex mechanism, varying according to the species, and even according to the different strains belonging to the same species. This is the case for *Escherichia coli*. Some strains show low levels of interaction with platelets and induce platelet aggregation with a low probability. Conversely, other strains, recognised with greater affinity by certain platelet receptors, may expose the patient to an increased thrombotic risk, due to their ability to induce platelet aggregation.

Antiplatelets may play an important role in the management of sepsis, particularly in *Escherichia coli*. However, although the use of this class of drugs in this indication has been widely published, almost none of these studies have evaluated the benefit of antiplatelet drugs in sepsis according to bacterial species.

In order to assess the pro-aggregation potential of *Escherichia coli* strains, the determination of specific markers present on *Escherichia coli* would allow the prediction of the capacity of this strain to interact with platelets. Thus, with regard to the potential role of the O antigen in these reactions, its *in-silico* serotyping would

allow the rapid determination of the serotype of the strain involved from the FASTA sequencing data (137,138).

The ability or inability to induce platelet aggregation for each of these strains could also be evaluated *in vitro*, in order to identify the strains and serotypes with a strong capacity to activate platelets. The identification of these strains would allow a more precise identification of the signalling pathways involved in platelet activation but would also make it possible to test each of the antiplatelet agents in order to assess which molecule is able to act in an optimal way to reduce platelet reactivity during sepsis.

Clinical studies are also needed to confirm the various results obtained *in vitro*, and to evaluate the clinical efficacy of antiplatelets in real-life conditions.

Figure Legends

Figure 1: Platelet-endothelium and platelet-neutrophil interactions.

Endothelial activation in response to infection induces platelet activation which in turn activates neutrophils through various signalling pathways.

CXCL4: platelet factor 4; PSGL-1: P-selectin glycoprotein 1; E-selectin: endothelial selectin.

Diagram created on biorender.com using SMART (Servier Medical Art).

Figure 2: Platelet response to LPS recognised (A) or not recognised (B) by CD14.

Upon full recognition of the LPS from the *Escherichia coli* strain, TLR4 will be able to complex with CD14 of soluble origin and be captured by platelets. This mechanism will result in the release of sCD40L present in platelet alpha granules, capable of activating GpIIbIIIa and responsible for platelet aggregation. Diagram created on biorender.com; sCD40L: soluble CD40L

Figure 3: Summary of intra-platelet signalling pathways secondary to interaction with *Escherichia coli*.

Depending on the strain involved, the signalling pathways involved in triggering platelet activation and aggregation may be different.

TLR4: Toll-like Receptor 4, PAF-R: Receptor of Platelet Activating Factor, FcγRIIA: Platelet Fcγ receptor, Akt: protein kinase B, PLA2: phospholipase A2, PLC: phospholipase C, p-LAT: phospho-LAT, PKC protein kinase C

Diagram created on biorender.com.

Table legends

Table 1 - Summary of data available in the literature concerning interactions between bacteria and platelets

726 **Table 1**

727

	Strains	Platelet receptor involved	Reference
Escherichia coli	O111	TLR4	(77)
	O157:H7	FcγRIIA	(80)
	O157	TLR4 CD62	(139)
	CFT073 (O6:H1)	FcγRIIA αIIbβ3 integrin	(72)
	RS218 (O18:H7:K1)	FcγRIIA αIIbβ3 integrin	(72)
	K12 C600	αIIbβ3 integrin TLR4	(25), (82), (86), (140), (141)
	O111:B4	TLR4	(140)
	O55	TLR4	(86)
Staphylococcus aureus		αIIbβ3 integrin GPIbα	(9,70)
Staphylococcus lugdunensis		αIIbβ3 integrin	(9,70)
Streptococcus pneumoniae		TLR2	(9,70)
Helicobacter pylori		vWF	(9,70)

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731 **References**

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734 1. Thomas M, Storey R. The role of platelets in inflammation. Thromb Haemost.
735 2015;114(09):449–58.
736 2. Singh A, Bisht P, Bhattacharya S, Guchhait P. Role of Platelet Cytokines in Dengue
737 Virus Infection. Front Cell Infect Microbiol. 2020 Sep 30;10:561366.
738 3. Tokarz-Deptuła B, Palma J, Baraniecki Ł, Stosik M, Kołacz R, Deptuła W. What
739 Function Do Platelets Play in Inflammation and Bacterial and Viral Infections? Front
740 Immunol. 2021 Dec 14;12:770436.
741 4. Katsounas A, Schlaak JF, Lempicki RA. CCL5: A Double-Edged Sword in Host
742 Defense Against the Hepatitis C Virus. International Reviews of Immunology. 2011 Nov
743 10;30(5–6):366–78.
744 5. Manne BK, Denorme F, Middleton EA, Portier I, Rowley JW, Stubben C, et al.
745 Platelet gene expression and function in patients with COVID-19. Blood. 2020 Sep
746 10;136(11):1317–29.
747 6. Campbell RA, Hisada Y, Denorme F, Grover SP, Bouck EG, Middleton EA, et al.
748 Comparison of the coagulopathies associated with COVID□19 and sepsis. Res Pract Thromb
749 Haemost [Internet]. 2021 May [cited 2022 Oct 8];5(4). Available from:
750 https://onlinelibrary.wiley.com/doi/10.1002/rth2.12525
751 7. Zhu A, Real F, Capron C, Rosenberg AR, Silvin A, Dunsmore G, et al. Infection of
752 lung megakaryocytes and platelets by SARS-CoV-2 anticipate fatal COVID-19. Cell Mol Life
753 Sci. 2022 Jul;79(7):365.
754 8. Aggrey AA, Srivastava K, Ture S, Field DJ, Morrell CN. Platelet Induction of the
755 Acute-Phase Response Is Protective in Murine Experimental Cerebral Malaria. JI. 2013 May
756 1;190(9):4685–91.
757 9. Cox D, Kerrigan SW, Watson SP. Platelets and the innate immune system:
758 mechanisms of bacterial-induced platelet activation. Journal of Thrombosis and Haemostasis.
759 2011;9(6):1097–107.
760 10. Ezzeroug Ezzraimi A, Hannachi N, Mariotti A, Rolland C, Levasseur A, Baron SA, et
761 al. The Antibacterial Effect of Platelets on Escherichia coli Strains. Biomedicines. 2022 Jun
762 28;10(7):1533.
763 11. Gotts JE, Matthay MA. Sepsis: pathophysiology and clinical management. BMJ. 2016
764 May 23;353:i1585.
765 12. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions
766 for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The
767 ACCP/SCCM Consensus Conference Committee. American College of Chest
768 Physicians/Society of Critical Care Medicine. Chest. 1992 Jun;101(6):1644–55.
769 13. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al.
770 The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).
771 JAMA. 2016 Feb 23;315(8):801–10.
772 14. Martinod K, Deppermann C. Immunothrombosis and thromboinflammation in host
773 defense and disease. Platelets. 2021 Apr 3;32(3):314–24.
774 15. Greco E, Lupia E, Bosco O, Vizio B, Montrucchio G. Platelets and Multi-Organ
775 Failure in Sepsis. Int J Mol Sci. 2017 Oct 20;18(10):E2200.
776 16. Koupenova M, Clancy L, Corkrey HA, Freedman JE. Circulating Platelets as
777 Mediators of Immunity, Inflammation, and Thrombosis. Circ Res. 2018 Jan 19;122(2):337–

778 51.
779 17. Asaduzzaman M, Lavasani S, Rahman M, Zhang S, Braun OÖ, Jeppsson B, et al.
780 Platelets support pulmonary recruitment of neutrophils in abdominal sepsis*: Critical Care
781 Medicine. 2009 Apr;37(4):1389–96.
782 18. Kaplan ZS, Jackson SP. The Role of Platelets in Atherothrombosis. Hematology. 2011
783 Dec 10;2011(1):51–61.
784 19. Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, et al. The
785 Glycoprotein Ib-IX-V Complex Is a Platelet Counterreceptor for P-Selectin. Journal of
786 Experimental Medicine. 1999 Sep 20;190(6):803–14.
787 20. Opal SM, van der Poll T. Endothelial barrier dysfunction in septic shock. J Intern
788 Med. 2015 Mar;277(3):277–93.
789 21. Shiraki R, Inoue N, Kawasaki S, Takei A, Kadotani M, Ohnishi Y, et al. Expression of
790 Toll-like receptors on human platelets. Thrombosis Research. 2004 Jan;113(6):379–85.
791 22. Andonegui G, Kerfoot SM, McNagny K, Ebbert KVJ, Patel KD, Kubes P. Platelets
792 express functional Toll-like receptor-4. Blood. 2005 Oct 1;106(7):2417–23.
793 23. Kraemer BF, Campbell RA, Schwertz H, Cody MJ, Franks Z, Tolley ND, et al. Novel
794 Anti-bacterial Activities of β-defensin 1 in Human Platelets: Suppression of Pathogen Growth
795 and Signaling of Neutrophil Extracellular Trap Formation. DeLeo FR, editor. PLoS Pathog.
796 2011 Nov 10;7(11):e1002355.
797 24. Semple JW, Aslam R, Kim M, Speck ER, Freedman J. Platelet-bound
798 lipopolysaccharide enhances Fc receptor-mediated phagocytosis of IgG-opsonized platelets.
799 Blood. 2007 Jun 1;109(11):4803–5.
800 25. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet
801 TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med.
802 2007 Apr;13(4):463–9.
803 26. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular Neutrophil
804 Extracellular Traps Capture Bacteria from the Bloodstream during Sepsis. Cell Host &
805 Microbe. 2012 Sep;12(3):324–33.
806 27. Sreeramkumar V, Adrover JM, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, et al.
807 Neutrophils scan for activated platelets to initiate inflammation. Science. 2014 Dec
808 5;346(6214):1234–8.
809 28. Goncalves R, Zhang X, Cohen H, Debrabant A, Mosser DM. Platelet activation
810 attracts a subpopulation of effector monocytes to sites of Leishmania major infection. Journal
811 of Experimental Medicine. 2011 Jun 6;208(6):1253–65.
812 29. Bakogiannis C, Sachse M, Stamatelopoulos K, Stellos K. Platelet-derived chemokines
813 in inflammation and atherosclerosis. Cytokine. 2019 Oct;122:154157.
814 30. Brown GT, Narayanan P, Li W, Silverstein RL, McIntyre TM. Lipopolysaccharide
815 Stimulates Platelets through an IL-1β Autocrine Loop. JI. 2013 Nov 15;191(10):5196–203.
816 31. Nording H, Langer HF. Complement links platelets to innate immunity. Seminars in
817 Immunology. 2018 Jun;37:43–52.
818 32. Palm F, Sjöholm K, Malmström J, Shannon O. Complement Activation Occurs at the
819 Surface of Platelets Activated by Streptococcal M1 Protein and This Results in Phagocytosis
820 of Platelets. JI. 2019 Jan 15;202(2):503–13.
821 33. Delabranche X, Stiel L, Severac F, Galoisy AC, Mauvieux L, Zobairi F, et al.
822 Evidence of Netosis in Septic Shock-Induced Disseminated Intravascular Coagulation. Shock.
823 2017 Mar;47(3):313–7.
824 34. Yang S, Qi H, Kan K, Chen J, Xie H, Guo X, et al. Neutrophil Extracellular Traps
825 Promote Hypercoagulability in Patients With Sepsis. Shock. 2017 Feb;47(2):132–9.
826 35. Denorme F, Campbell RA. Procoagulant platelets: novel players in
827 thromboinflammation. Am J Physiol Cell Physiol. 2022 Oct 1;323(4):C951–8.

36. Zaid Y, Merhi Y. Implication of Platelets in Immuno-Thrombosis and Thrombo-Inflammation. Front Cardiovasc Med. 2022 Mar 25;9:863846.
37. Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood. 2014 May 1;123(18):2768–76.
38. Gómez H, Kellum JA. Sepsis-induced acute kidney injury. Curr Opin Crit Care. 2016 Dec;22(6):546–53.
39. Yan J, Li S, Li S. The role of the liver in sepsis. Int Rev Immunol. 2014 Dec;33(6):498–510.
40. Vincent JL, Zhang H, Szabo C, Preiser JC. Effects of nitric oxide in septic shock. Am J Respir Crit Care Med. 2000 Jun;161(6):1781–5.
41. Palace SG, Vitseva O, Proulx MK, Freedman JE, Goguen JD, Koupenova M. *Yersinia pestis* escapes entrapment in thrombi by targeting platelet function. J Thromb Haemost. 2020 Dec;18(12):3236–48.
42. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. Nat Rev Immunol. 2008 Oct;8(10):776–87.
43. Levi M, Ten Cate H. Disseminated intravascular coagulation. N Engl J Med. 1999 Aug 19;341(8):586–92.
44. Donzé JD, Ridker PM, Finlayson SRG, Bates DW. Impact of sepsis on risk of postoperative arterial and venous thromboses: large prospective cohort study. BMJ. 2014 Sep 8;349:g5334.
45. Larkin CM, Santos-Martinez MJ, Ryan T, Radomski MW. Sepsis-associated thrombocytopenia. Thrombosis Research. 2016 May 1;141:11–6.
46. Vanderschueren S, De Weerdt A, Malbrain M, Vankersschaever D, Frans E, Wilmer A, et al. Thrombocytopenia and prognosis in intensive care: Critical Care Medicine. 2000 Jun;28(6):1871–6.
47. Venkata C, Kashyap R, Farmer JC, Afessa B. Thrombocytopenia in adult patients with sepsis: incidence, risk factors, and its association with clinical outcome. j intensive care. 2013 Dec;1(1):9.
48. Dewitte A, Lepreux S, Villeneuve J, Rigothier C, Combe C, Ouattara A, et al. Blood platelets and sepsis pathophysiology: A new therapeutic prospect in critical ill patients? Ann Intensive Care. 2017 Dec 1;7(1):115.
49. Bonten M, Johnson JR, van den Biggelaar AHJ, Georgalis L, Geurtsen J, de Palacios PI, et al. Epidemiology of Escherichia coli Bacteremia: A Systematic Literature Review. Clin Infect Dis. 2021 Apr 8;72(7):1211–9.
50. Heal Protection Report. Laboratory surveillance of Escherichia coli bacteraemia in England, Wales and Northern Ireland: 2018 [Internet]. Public Health England; 2019 Oct p. 15. (Volume 13). Report No.: 37. Available from: https://assets.publishing.service.gov.uk/
51. Kjellander C, Björkholm M, Källman O, Giske CG, Weibull CE, Löve TJ, et al. Bloodstream infections in patients with chronic lymphocytic leukemia: a longitudinal single-center study. Ann Hematol. 2016 May;95(6):871–9.
52. Cattaneo C, Antoniazzi F, Tumbarello M, Skert C, Borlenghi E, Schieppati F, et al. Relapsing bloodstream infections during treatment of acute leukemia. Ann Hematol. 2014 May;93(5):785–90.
53. Teh BW, Harrison SJ, Slavin MA, Worth LJ. Epidemiology of bloodstream infections in patients with myeloma receiving current era therapy. Eur J Haematol. 2017 Feb;98(2):149–53.
54. Trecarichi EM, Pagano L, Candoni A, Pastore D, Cattaneo C, Fanci R, et al. Current epidemiology and antimicrobial resistance data for bacterial bloodstream infections in patients with hematologic malignancies: an Italian multicentre prospective survey. Clinical Microbiology and Infection. 2015 Apr;21(4):337–43.

55. Marín M, Gudiol C, Garcia-Vidal C, Ardanuy C, Carratalà J. Bloodstream Infections in Patients With Solid Tumors: Epidemiology, Antibiotic Therapy, and Outcomes in 528 Episodes in a Single Cancer Center. Medicine. 2014 May;93(3):143–9.
56. Belhassen-García M, Velasco-Tirado V, López-Bernus A, Alonso-Sardón M, Carpio-Pérez A, Fuentes-Pardo L, et al. Fever of unknown origin as the first manifestation of colonic pathology. Clin Med. 2013 Apr;13(2):141–5.
57. Jannasch O, Kelch B, Adolf D, Tammer I, Lodes U, Weiss G, et al. Nosocomial Infections and Microbiologic Spectrum after Major Elective Surgery of the Pancreas, Liver, Stomach, and Esophagus. Surgical Infections. 2015 Jun;16(3):338–45.
58. Hizette P, Simoens C, Massaut J, Thill V, Smets D, da Costa PM. Septic shock in digestive surgery: a retrospective study of 89 patients. Hepatogastroenterology. 2009 Dec;56(96):1615–21.
59. Drew RJ, Fonseca-Kelly Z, Eogan M. A Retrospective Audit of Clinically Significant Maternal Bacteraemia in a Specialist Maternity Hospital from 2001 to 2014. Infectious Diseases in Obstetrics and Gynecology. 2015;2015:1–4.
60. Weston EJ, Pondo T, Lewis MM, Martell-Cleary P, Morin C, Jewell B, et al. The Burden of Invasive Early-onset Neonatal Sepsis in the United States, 2005–2008. Pediatric Infectious Disease Journal. 2011 Nov;30(11):937–41.
61. Simonsen KA, Anderson-Berry AL, Delair SF, Davies HD. Early-Onset Neonatal Sepsis. Clin Microbiol Rev. 2014 Jan;27(1):21–47.
62. Kaper JB, Nataro JP, Mobley HL. Pathogenic Escherichia coli. Nat Rev Microbiol. 2004 Feb;2(2):123–40.
63. Lüthje P, Brauner A. Virulence factors of uropathogenic E. coli and their interaction with the host. Adv Microb Physiol. 2014;65:337–72.
64. Poirel L, Madec JY, Lupo A, Schink AK, Kieffer N, Nordmann P, et al. Antimicrobial Resistance in Escherichia coli. Microbiol Spectr. 2018 Jul;6(4).
65. Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U, Cantón R, et al. Escherichia coli: an old friend with new tidings. FEMS Microbiol Rev. 2016 Jul 1;40(4):437–63.
66. Herrmann M, Lai QJ, Albrecht RM, Mosher DF, Proctor RA. Adhesion of Staphylococcus aureus to surface-bound platelets: role of fibrinogen/fibrin and platelet integrins. J Infect Dis. 1993 Feb;167(2):312–22.
67. Bayer AS, Sullam PM, Ramos M, Li C, Cheung AL, Yeaman MR. Staphylococcus aureus induces platelet aggregation via a fibrinogen-dependent mechanism which is independent of principal platelet glycoprotein IIb/IIIa fibrinogen-binding domains. Infect Immun. 1995 Sep;63(9):3634–41.
68. Kurpiewski GE, Forrester LJ, Campbell BJ, Barrett JT. Platelet aggregation by Streptococcus pyogenes. Infect Immun. 1983 Feb;39(2):704–8.
69. Keane C, Tilley D, Cunningham A, Smolenski A, Kadioglu A, Cox D, et al. Invasive Streptococcus pneumoniae trigger platelet activation via Toll-like receptor 2. J Thromb Haemost. 2010 Dec;8(12):2757–65.
70. Cox D. Bacteria–platelet interactions. Journal of Thrombosis and Haemostasis. 2009 Nov;7(11):1865–6.
71. Sharda A, Flaumenhaft R. The life cycle of platelet granules. F1000Res. 2018 Feb 28;7:236.
72. Watson CN, Kerrigan SW, Cox D, Henderson IR, Watson SP, Arman M. Human platelet activation by Escherichia coli: roles for FcγRIIA and integrin αIIbβ3. Platelets. 2016 Sep;27(6):535–40.
73. Ezzeroug Ezzraimi A, Hannachi N, Mariotti A, Rolain JM, Camoin-Jau L. Platelets and Escherichia coli: A Complex Interaction. Biomedicines. 2022 Jul 7;10(7):1636.

74. Sheu, Hung, Kan, Lee, Yen. Mechanisms involved in the antiplatelet activity of *Escherichia coli* lipopolysaccharide in human platelets: LPS inhibits Platelet Aggregation in Washed Human Platelets. British Journal of Haematology. 1998 Oct;103(1):29–38.
75. Roka-Moiia Y, Walk R, Palomares DE, Ammann KR, Dimasi A, Italiano JE, et al. Platelet Activation via Shear Stress Exposure Induces a Differing Pattern of Biomarkers of Activation versus Biochemical Agonists. Thromb Haemost. 2020 May;120(05):776–92.
76. Shannon O. Determining Platelet Activation and Aggregation in Response to Bacteria. In: Nordenfelt P, Collin M, editors. Bacterial Pathogenesis [Internet]. New York, NY: Springer New York; 2017 [cited 2022 Jul 28]. p. 267–73. (Methods in Molecular Biology; vol. 1535). Available from: http://link.springer.com/10.1007/978-1-4939-6673-8_17
77. Petruzziello-Pellegrini TN, Moslemi-Naeini M, Marsden PA. New insights into Shiga toxin-mediated endothelial dysfunction in hemolytic uremic syndrome. Virulence. 2013 Aug 15;4(6):556–63.
78. Matus V, Valenzuela JG, Hidalgo P, Pozo LM, Panes O, Wozniak A, et al. Human platelet interaction with E. coli O111 promotes tissue-factor-dependent procoagulant activity, involving Toll like receptor 4. PLoS One. 2017;12(9):e0185431.
79. Zhao L, Ohtaki Y, Yamaguchi K, Matsushita M, Fujita T, Yokochi T, et al. LPS-induced platelet response and rapid shock in mice: contribution of O-antigen region of LPS and involvement of the lectin pathway of the complement system. Blood. 2002 Nov 1;100(9):3233–9.
80. Moriarty RD, Cox A, McCall M, Smith SGJ, Cox D. Escherichia coli induces platelet aggregation in an FcγRIIa-dependent manner. J Thromb Haemost. 2016 Apr;14(4):797–806.
81. Clawson CC, White JG. Platelet Interaction with Bacteria. Am J Pathol. 1971 Nov;65(2):367–80.
82. Fejes AV, Best MG, van der Heijden WA, Vancura A, Verschueren H, de Mast Q, et al. Impact of Escherichia coli K12 and O18:K1 on human platelets: Differential effects on platelet activation, RNAs and proteins. Sci Rep. 2018 Nov 1;8(1):16145.
83. Pupo E, Lindner B, Brade H, Schromm AB. Intact rough□ and smooth□form lipopolysaccharides from *E scherichia coli* separated by preparative gel electrophoresis exhibit differential biologic activity in human macrophages. FEBS J. 2013 Feb;280(4):1095–111.
84. Vallance TM, Zeuner MT, Williams HF, Widera D, Vaiyapuri S. Toll-Like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis. Mediators Inflamm. 2017;2017:9605894.
85. Berthet J, Damien P, Hamzeh-Cognasse H, Pozzetto B, Garraud O, Cognasse F. Toll-like receptor 4 signal transduction in platelets: novel pathways: Correspondence. British Journal of Haematology. 2010 Jul 7;151(1):89–92.
86. Zhang G, Han J, Welch EJ, Ye RD, Voyno-Yasenetskaya TA, Malik AB, et al. Lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway. J Immunol. 2009 Jun 15;182(12):7997–8004.
87. Niklaus M, Klingler P, Weber K, Koessler A, Boeck M, Kobsar A, et al. The involvement of toll-like receptors 2 and 4 in human platelet signalling pathways. Cellular Signalling. 2020 Dec;76:109817.
88. Wang E, Liu Y, Qiu X, Tang Y, Wang H, Xiao X, et al. Bacteria-released outer membrane vesicles promote disseminated intravascular coagulation. Thrombosis Research. 2019 Jun;178:26–33.
89. Jerez-Dolz D, Torramade-Moix S, Palomo M, Moreno-Castaño A, Lopez-Vilchez I, Hernandez R, et al. Internalization of microparticles by platelets is partially mediated by toll-like receptor 4 and enhances platelet thrombogenicity. Atherosclerosis. 2020 Feb;294:17–24.

90. Grover SP, Mackman N. Tissue Factor: An Essential Mediator of Hemostasis and Trigger of Thrombosis. ATVB. 2018 Apr;38(4):709–25.
91. Morrissey JH, Drake TA. Procoagulant Response of the Endothelium and Monocytes. In: Schlag G, Redl H, editors. Pathophysiology of Shock, Sepsis, and Organ Failure [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 1993 [cited 2022 Oct 20]. p. 564–74. Available from: http://link.springer.com/10.1007/978-3-642-76736-4_41
92. Berthet J, Damien P, Hamzeh-Cognasse H, Arthaud CA, Eyraud MA, Zéni F, et al. Human platelets can discriminate between various bacterial LPS isoforms via TLR4 signaling and differential cytokine secretion. Clinical Immunology. 2012 Dec;145(3):189–200.
93. Damien P, Cognasse F, Eyraud MA, Arthaud CA, Pozzetto B, Garraud O, et al. LPS stimulation of purified human platelets is partly dependent on plasma soluble CD14 to secrete their main secreted product, soluble-CD40-Ligand. BMC Immunol. 2015 Dec;16(1):3.
94. Thorgersen EB, Hellerud BC, Nielsen EW, Barratt□Due A, Fure H, Lindstad JK, et al. CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses in *Escherichia coli* sepsis in pigs. FASEB j. 2010 Mar;24(3):712–22.
95. Keshari RS, Silasi R, Popescu NI, Regmi G, Chaaban H, Lambris JD, et al. CD14 inhibition improves survival and attenuates thrombo□inflammation and cardiopulmonary dysfunction in a baboon model of *Escherichia coli* sepsis. J Thromb Haemost. 2021 Feb;19(2):429–43.
96. Davì G, Ferroni P. CD40-CD40L interactions in platelet activation. Thromb Haemost. 2005;93(06):1011–2.
97. Kojok K, Mohsen M, El Kadiry AEH, Mourad W, Merhi Y. Aspirin Reduces the Potentiating Effect of CD40L on Platelet Aggregation via Inhibition of Myosin Light Chain. JAHA. 2020 Feb 4;9(3):e013396.
98. Abhilasha KV, Sumanth MS, Chaithra VH, Jacob SP, Thyagarajan A, Sahu RP, et al. p38 MAP-kinase inhibitor protects against platelet-activating factor-induced death in mice. Free Radical Biology and Medicine. 2019 Nov;143:275–87.
99. Wang H, Tan X di, Chang H, Gonzalez-Crussi F, Remick DG, Hsueh W. Regulation of platelet-activating factor receptor gene expression *in vivo* by endotoxin, platelet-activating factor and endogenous tumour necrosis factor. Biochemical Journal. 1997 Mar 1;322(2):603–8.
100. Abhilasha KV, Sumanth MS, Thyagarajan A, Sahu RP, Kemparaju K, Marathe GK. Reversible cross-tolerance to platelet-activating factor signaling by bacterial toxins. Platelets. 2021 Oct 3;32(7):960–7.
101. Riaz AH, Tasma BE, Woodman ME, Wooten RM, Worth RG. Human platelets efficiently kill IgG-opsonized *E. coli*. FEMS Immunol Med Microbiol. 2012 Jun;65(1):78–83.
102. Arman M, Krauel K. Human platelet IgG Fc receptor FcγRIIA in immunity and thrombosis. J Thromb Haemost. 2015 Jun;13(6):893–908.
103. Piotti A, Novelli D, Meessen JMTA, Ferlicca D, Coppolecchia S, Marino A, et al. Endothelial damage in septic shock patients as evidenced by circulating syndecan-1, sphingosine-1-phosphate and soluble VE-cadherin: a substudy of ALBIOS. Crit Care. 2021 Mar 19;25(1):113.
104. Joffre J, Hellman J, Ince C, Ait-Oufella H. Endothelial Responses in Sepsis. Am J Respir Crit Care Med. 2020 Aug 1;202(3):361–70.
105. Ince C, Mayeux PR, Nguyen T, Gomez H, Kellum JA, Ospina-Tascón GA, et al. The Endothelium in Sepsis. Shock. 2016 Mar;45(3):259–70.
106. Yeaman MR. Platelets: at the nexus of antimicrobial defence. Nat Rev Microbiol. 2014 Jun;12(6):426–37.
107. Li C, Li J, Ni H. Crosstalk Between Platelets and Microbial Pathogens. Front Immunol. 2020 Aug 7;11:1962.

108. Aquino-Domínguez AS, Romero-Tlalolini M de LA, Torres-Aguilar H, Aguilar-Ruiz SR. Recent Advances in the Discovery and Function of Antimicrobial Molecules in Platelets. Int J Mol Sci. 2021 Sep 23;22(19):10230.
109. Krijgsveld J, Zaat SA, Meeldijk J, van Veelen PA, Fang G, Poolman B, et al. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. J Biol Chem. 2000 Jul 7;275(27):20374–81.
110. Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. Crit Rev Biotechnol. 2012 Jun;32(2):143–71.
111. Baron S, Hadjadj L, Rolain JM, Olaitan AO. Molecular mechanisms of polymyxin resistance: knowns and unknowns. Int J Antimicrob Agents. 2016 Dec;48(6):583–91.
112. Assinger A, Schrottmaier WC, Salzmann M, Rayes J. Platelets in Sepsis: An Update on Experimental Models and Clinical Data. Front Immunol. 2019 Jul 17;10:1687.
113. Hui P, Cook DJ, Lim W, Fraser GA, Arnold DM. The Frequency and Clinical Significance of Thrombocytopenia Complicating Critical Illness. Chest. 2011 Feb;139(2):271–8.
114. Thiolliere F, Serre-Sapin AF, Reignier J, Benedit M, Constantin JM, Lebert C, et al. Epidemiology and outcome of thrombocytopenic patients in the intensive care unit: results of a prospective multicenter study. Intensive Care Med. 2013 Aug;39(8):1460–8.
115. Aslam R, Speck ER, Kim M, Crow AR, Bang KWA, Nestel FP, et al. Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-α production in vivo. Blood. 2006 Jan 15;107(2):637–41.
116. Trauer J, Muhi S, McBryde ES, Al Harbi SA, Arabi YM, Boyle AJ, et al. Quantifying the Effects of Prior Acetyl-Salicylic Acid on Sepsis-Related Deaths: An Individual Patient Data Meta-Analysis Using Propensity Matching. Crit Care Med. 2017 Nov;45(11):1871–9.
117. Ouyang Y, Wang Y, Liu B, Ma X, Ding R. Effects of antiplatelet therapy on the mortality rate of patients with sepsis: A meta-analysis. J Crit Care. 2019 Apr 1;50:162–8.
118. Erlich JM, Talmor DS, Cartin-Ceba R, Gajic O, Kor DJ. Prehospitalization Antiplatelet Therapy Is Associated With a Reduced Incidence of Acute Lung Injury. Chest. 2011 Feb;139(2):289–95.
119. Kor DJ, Carter RE, Park PK, Festic E, Banner-Goodspeed VM, Hinds R, et al. Effect of Aspirin on Development of ARDS in At-Risk Patients Presenting to the Emergency Department: The LIPS-A Randomized Clinical Trial. JAMA. 2016 Jun 14;315(22):2406.
120. Lösche W, Boettel J, Kabisch B, Winning J, Claus RA, Bauer M. Do aspirin and other antiplatelet drugs reduce the mortality in critically ill patients? Thrombosis. 2012;2012:720254.
121. Falcone M, Russo A, Cangemi R, Farcomeni A, Calvieri C, Barillà F, et al. Lower Mortality Rate in Elderly Patients With Community□Onset Pneumonia on Treatment With Aspirin. JAHA. 2015 Jan 5;4(1):e001595.
122. Wang Y, Ouyang Y, Liu B, Ma X, Ding R. Platelet activation and antiplatelet therapy in sepsis: A narrative review. Thromb Res. 2018 Jun;166:28–36.
123. Eisen DP, Reid D, McBryde ES. Acetyl salicylic acid usage and mortality in critically ill patients with the systemic inflammatory response syndrome and sepsis. Crit Care Med. 2012 Jun;40(6):1761–7.
124. Campbell R, McGuire A, Young L, Mackay A. Aspirin and statin therapy in sepsis, a red herring? Intensive Care Med Exp. 2015 Oct 1;3(Suppl 1):A227.
125. Al Harbi SA, Tamim HM, Al-Dorzi HM, Sadat M, Arabi YM. Association between aspirin therapy and the outcome in critically ill patients: a nested cohort study. BMC Pharmacology and Toxicology. 2016 Feb 5;17(1):5.
126. Hannachi N, Ogé-Ganaye E, Baudoin JP, Fontanini A, Bernot D, Habib G, et al. Antiplatelet Agents Have a Distinct Efficacy on Platelet Aggregation Induced by Infectious

Bacteria. Front Pharmacol. 2020 Jun 5;11:863.
127. Osthoff M, Sidler JA, Lakatos B, Frei R, Dangel M, Weisser M, et al. Low-Dose Acetylsalicylic Acid Treatment and Impact on Short-Term Mortality in Staphylococcus aureus Bloodstream Infection: A Propensity Score-Matched Cohort Study. Crit Care Med. 2016 Apr;44(4):773–81.
128. Kiers D, van der Heijden WA, van Ede L, Gerretsen J, de Mast Q, van der Ven AJ, et al. A randomised trial on the effect of anti-platelet therapy on the systemic inflammatory response in human endotoxaemia. Thromb Haemost. 2017 Aug 30;117(9):1798–807.
129. Judge HM, Buckland RJ, Holgate CE, Storey RF. Glycoprotein IIb/IIIa and P2Y $_{12}$ receptor antagonists yield additive inhibition of platelet aggregation, granule secretion, soluble CD40L release and procoagulant responses. Platelets. 2005 Nov;16(7):398–407.
130. Gremmel T, Frelinger AL, Michelson AD. Soluble CD40 Ligand in Aspirin-Treated Patients Undergoing Cardiac Catheterization. Ahrens I, editor. PLoS ONE. 2015 Aug 3;10(8):e0134599.
131. Vardon-Bounes F, Garcia C, Piton A, Series J, Gratacap MP, Poëtte M, et al. Evolution of Platelet Activation Parameters During Septic Shock in Intensive Care Unit. Platelets. 2021 Dec 16;1–8.
132. Lorente L, Martín MM, Varo N, Borreguero-León JM, Solé-Violán J, Blanquer J, et al. Association between serum soluble CD40 ligand levels and mortality in patients with severe sepsis. Crit Care. 2011;15(2):R97.
133. de Souza Brito F, Tricoci P. Novel Anti-platelet Agents: Focus on Thrombin Receptor Antagonists. J of Cardiovasc Trans Res. 2013 Jun;6(3):415–24.
134. Schoergenhofer C, Schwameis M, Gelbenegger G, Buchtele N, Thaler B, Mussbacher M, et al. Inhibition of Protease-Activated Receptor (PAR1) Reduces Activation of the Endothelium, Coagulation, Fibrinolysis and Inflammation during Human Endotoxemia. Thromb Haemost. 2018 Jul;118(07):1176–84.
135. Derhaschnig U, Bergmair D, Marsik C, Schlifke I, Wijdenes J, Jilma B. Effect of interleukin-6 blockade on tissue factor-induced coagulation in human endotoxemia: Critical Care Medicine. 2004 May;32(5):1136–40.
136. Pernerstorfer T, Hollenstein U, Hansen JB, Knechtelsdorfer M, Stohlawetz P, Graninger W, et al. Heparin Blunts Endotoxin-Induced Coagulation Activation. Circulation. 1999 Dec 21;100(25):2485–90.
137. Joensen KG, Tetzschner AMM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and Easy *In Silico* Serotyping of Escherichia coli Isolates by Use of Whole-Genome Sequencing Data. Carroll KC, editor. J Clin Microbiol. 2015 Aug;53(8):2410–26.
138. Bessonov K, Laing C, Robertson J, Yong I, Ziebell K, Gannon VPJ, et al. ECTyper: in silico Escherichia coli serotype and species prediction from raw and assembled whole-genome sequence data. Microbial Genomics [Internet]. 2021 Dec 3 [cited 2022 Jul 1];7(12). Available from: https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000728
139. Ståhl A lie, Sartz L, Nelsson A, Békássy ZD, Karpman D. Shiga toxin and lipopolysaccharide induce platelet-leukocyte aggregates and tissue factor release, a thrombotic mechanism in hemolytic uremic syndrome. PLoS One. 2009 Sep 11;4(9):e6990.
140. Vallance TM, Ravishankar D, Albadawi DAI, Layfield H, Sheard J, Vaiyapuri R, et al. Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation. Sci Rep. 2019 Dec;9(1):18258.
141. Lopes Pires ME, Clarke SR, Marcondes S, Gibbins JM. Lipopolysaccharide potentiates platelet responses via toll-like receptor 4-stimulated Akt-Erk-PLA2 signalling. Ahrens I, editor. PLoS ONE. 2017 Nov 14;12(11):e0186981.

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Figure 1.JPEG

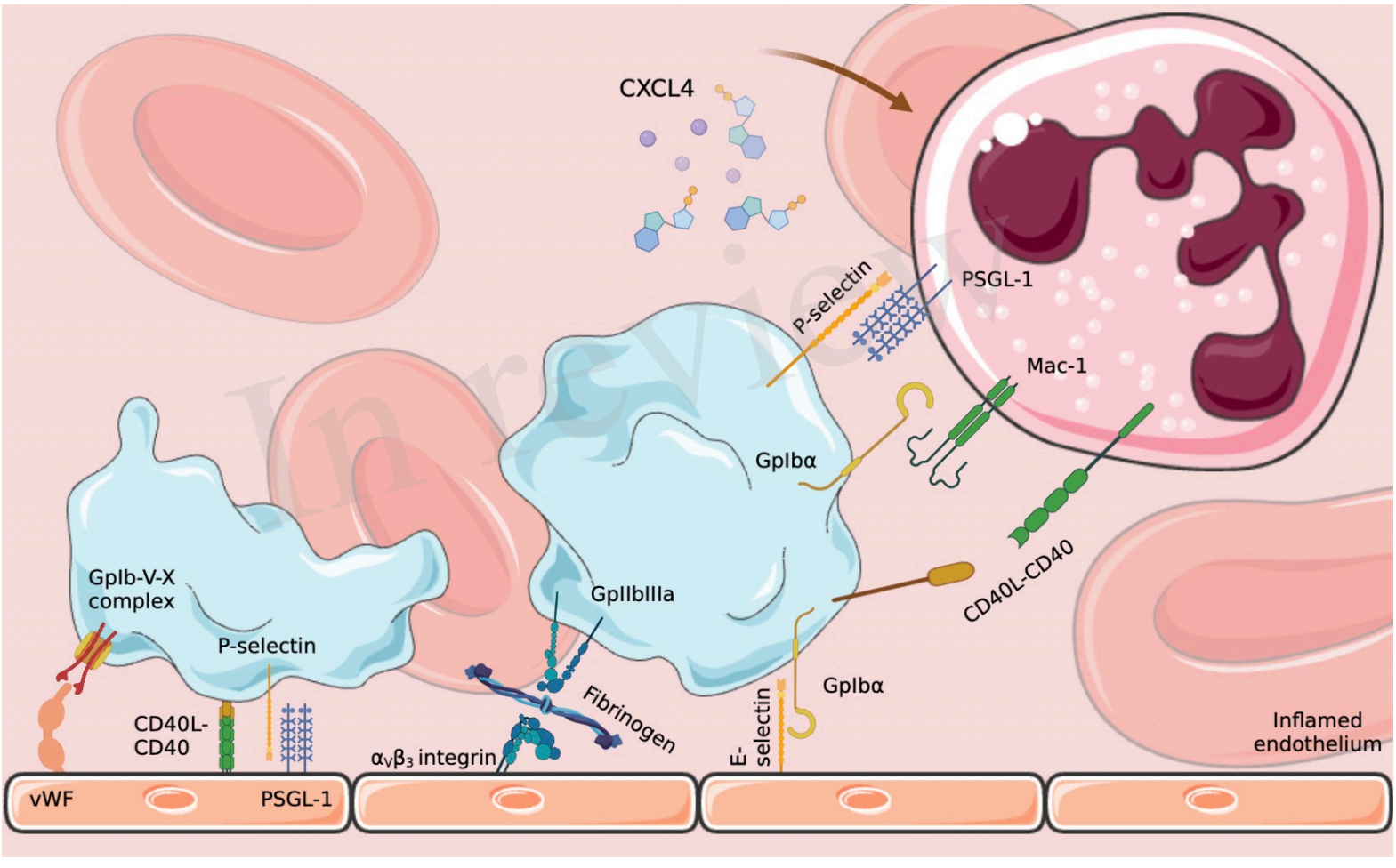


Figure 2.JPEG

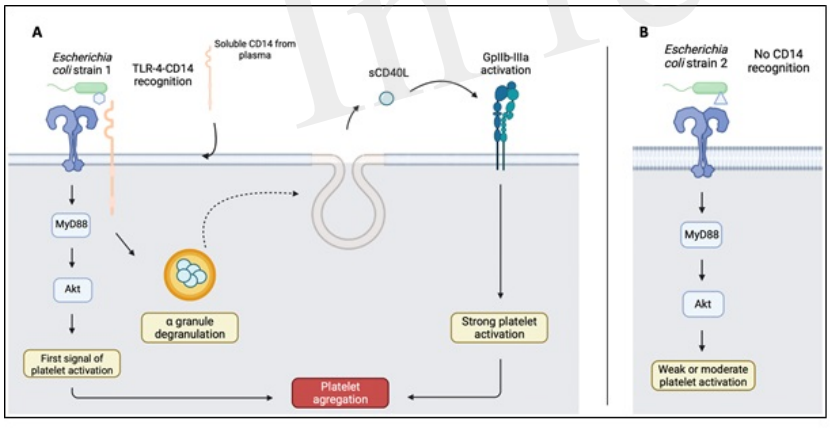
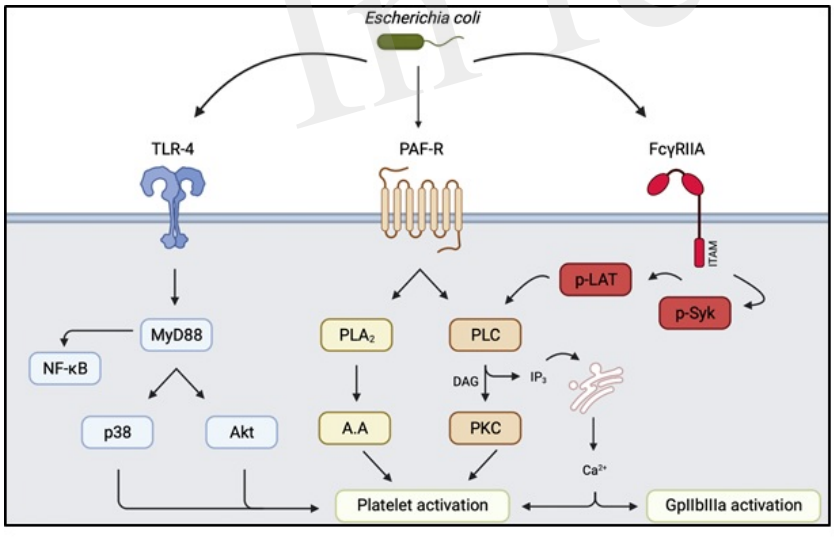


Figure 3.JPEG



Préambule

Bien que *E. coli* soit impliqué dans des infections graves au cours desquelles des phénomènes d'immunothromboses sont décrits, les phénomènes d'agrégation plaquettaire induits par des bactéries sont principalement étudiés sur bactéries à Gram-positif, dont les staphylocoques tels que *Staphylococcus aureus* et *Staphylococcus epidermidis* et les streptocoques tels que *Streptococcus sanguinis* et *Streptococcus gordonii* (58,59). Très peu de travaux *in vitro* ont caractérisé le potentiel pro-agrégant d'un large panel de souches de *E. coli.*

Le but de notre étude était d'étudier l'agrégation plaquettaire médiée par 10 souches de *E. coli* en utilisant cinq souches résistantes à la colistine provenant d'isolats cliniques et cinq souches de référence, sensibles à la colistine. La capacité de ces dix souches à induire l'agrégation plaquettaire a été évaluée par deux méthodes complémentaires : la spectrophotométrie et la microscopie confocale. L'effet AAP a été évalué sur les souches qui induisaient l'agrégation plaquettaire.

Nos résultats démontrent que parmi les dix souches testées, à trois concentrations bactériennes différentes, seulement quatre d'entre elles étaient capables d'induire une agrégation plaquettaire significative. Cette agrégation est concentration dépendante pour trois souches. Seule la souche J53 induit une agrégation significative avec les trois concentrations bactériennes testées. Nous avons émis l'hypothèse que cette différence de réponse est due à la structure des antigènes-O différents. L'analyse *in silico* des différents sérotypes de l'antigène-O montre que deux souches ayant le même sérotype présentent les mêmes profils d'agrégation. De manière intéressante, bien que les souches apparentées J53 et K12 présentent le même sérotype, elles ont des effets inverses sur l'agrégation plaquettaire. La souche J53 induit une forte agrégation plaquettaire, qui n'est pas retrouvée avec la souche K12. Ce comportement opposé de ces souches serait la conséquence de la délétion de l'élément mobile IS5 dans le gène *wbbL* de la souche J53 lui permettant d'exprimer un antigène-O fonctionnel.

L'analyse par microscopie confocale révèle de nombreux agrégats contenant des bacilles avec les souches qui induisent une forte agrégation plaquettaire. Ces observations confirment les résultats obtenus par spectrophotométrie. A l'inverse, le nombre de bacilles observés est plus faible en présence des souches qui n'induisent pas d'agrégats plaquettaires comme observé par spectrophotométrie.

L'évaluation de l'effet des AAP sur l'agrégation a été principalement réalisée sur la souche *E. coli* J53, responsable d'une forte agrégation plaquettaire. Le traitement des plaquettes avec un AAP n'inhibe pas complètement la capacité des plaquettes à s'agréger. L'association

de ces deux molécules ne permet pas d'obtenir un effet synergique, et ne présenterait pas de bénéfice par rapport à l'utilisation de ticagrelor seul. Cette persistance de l'agrégation pourrait être due au mécanisme d'activation des plaquettes par des voies de signalisation non classiques. Le TLR-4 serait capable de reconnaître le LPS de différentes souches de manière hétérogène. Certains LPS se lierait de manière exclusive au TLR-4, tandis que d'autres seraient capable d'induire le recrutement à partir du plasma de CD14 soluble (sCD14) et d'induire la formation d'un complexe TLR-4-sCD14-LPS, mettant en jeu des voies de signalisation spécifiques aboutissant à la libération de CD40L soluble (sCD40L) (60,61). Le CD40L soluble (sCD40L), libéré à partir des granules plaquettaires en réponse à cette stimulation par le complexe TLR-4-sCD14-LPS (61), est capable d'induire une augmentation de la sécrétion du thromboxane (TxA2) (62), et de potentialiser la capacité des plaquettes à s'agréger en se liant à l'intégrine αIIbβ3 (63), expliquant la persistance d'une agrégation plaquettaire qui court-circuite les voies classiques d'agrégation ciblées par l'aspirine et les inhibiteurs de P_2Y_{12}. Lorsqu'on s'intéresse aux niveaux de CD40L chez les patients traités par différents antiplaquettaires, les taux CD40L ne sont effectivement pas significativement modifiés par la prise d'aspirine, alors que ces taux sont abaissés lors de la prise associée d'inhibiteur de P_2Y_{12} (64). Cette différence pourrait expliquer pourquoi le ticagrelor permet une meilleure réduction de l'agrégation plaquettaire.

Nos travaux démontrent que le profil d'agrégation plaquettaire induit par les souches d'*E. coli* serait indépendant de l'état de résistance à la colistine mais serait sous la dépendance de la structure du LPS. Les AAP testés permettent de diminuer l'agrégation plaquettaire observée induite par certaines souches sans l'inhiber totalement

Article 5: Effect of antiplatelets on the interactions between blood platelets and *Escherichia coli* strains

Mariotti, A.; **Ezzeroug Ezzraimi, A**.; Baudoin, J-P.; Camoin-Jau, L.

(En cours de rédaction)

Effect of antiplatelets on the interactions between blood platelets and *Escherichia coli* strains

Antoine Mariotti[1,2,3] , Amina Ezzeroug Ezzraimi[1,2] , Jean-Pierre Baudoin[1,2] , Laurence Camoin-Jau[1,2,3]

[1]Aix Marseille Univ, IRD, APHM, MEPHI, IHU Méditerranée infection, Marseille, France

[2]IHU Méditerranée Infection, Boulevard Jean Moulin, 13385 Marseille, France

[3]Haematology Laboratory, Hôpital de la Timone, APHM, Boulevard Jean- Moulin, 13385 Marseille, France

[*]**Corresponding author:**
Prof. Laurence Camoin-Jau
Aix Marseille University, IRD, MEPHI, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France.
Tel: 33 4 13 73 24 01
Fax: 33 4 13 73 24 02
Email: laurence.camoin@ap-hm.fr

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Abstract

Background: Despite the ability of *Escherichia coli* to induce platelet activation and aggregation, the mechanisms of this interaction are still not clearly established. The aim of our study was to investigate platelet aggregation mediated by *Escherichia coli* strains and the effect of antiplatelet drugs in this phenomenom.

Materials and methods: Ten *Escherichia coli* strains *were* used, five colistin-resistant strains from clinical isolates, and five reference strains, sensitive to colistin, available in the CSUR collection of the IHU Mediterrannée Infection. The ability of these ten strains to induce platelet aggregation was evaluated by two complementary methods: spectrophometry and confocal microscopy. The effect of antiplatelet drugs was evaluated on the strains responsible for platelet aggregation.

Results: Among the ten strains tested, only four have induced a significant aggregation. This variability observed in aggregometry was found in confocal microscopy. Four strains were able to induce the formation of large platelet aggregates in which the bacilli are found. In presence of the other strains, platelets had remained in a non-activated state despite the proximity to the bacteria. Treatment of platelets with an antiplatelet agent did not completely suppress the platelet aggregations.

Conclusion: Aggregation induced by *Escherichia coli* is strain and concentration dependent. These differences in behavior are most likely related to differences in the structure of the LPS of the bacteria, and in particular the O antigen.

Introduction

In addition to their action in haemostasis, platelets play a major role in the anti-infective response, as a result of an interaction between bacteria and platelets. These interactions can trigger platelet activation, leading to the release of granular contents and the secretion of molecules with multiple roles: direct bactericidal effect, chemotaxis, leukocyte-platelet interaction.... The ability of platelets to interact with bacteria involves several types of mechanisms that vary according to the bacterial species. Indeed, platelets are equipped with numerous receptors, such as TLR-4 (1) which allow them to recognise certain bacterial motifs, and more particularly in the case of gram-negative bacilli, to interact with lipopolysaccharide (LPS) (2). *Escherichia coli* is a Gram-negative commensal bacterium, belonging to the Enterobacteriaceae family, which is very frequently found in the human digestive tract and represents a large part of the intestinal flora. Certain strains are often found in human pathology, particularly in community and nosocomial infections, such as meningitis, gastroenteritis, urinary tract infections, etc. (3). *Escherichia coli* strains are capable of acquiring virulence factors (adhesins, capsule, synthesis and secretion of toxins, etc.) which give them pathogenic power, as well as mechanisms of resistance to antibiotics which give them reduced sensitivity to certain anti-infectious molecules (4,5). Resistance to colistin, although rare in *Escherichia coli*, results, for the most part, from the acquisition by horizontal transfer of a gene from the *mcr* family, and more particularly *mcr-1* (6) which modifies the structure of lipid A of LPS. This LPS structure of colistin-resistant *Escherichia coli* strains could modify the interactions between these pathogens and platelets.

Although *Escherichia coli* is frequently encountered in clinical practice, studies of interactions leading to platelet activation have focused on Gram-positive bacteria, including staphylococci (*Staphylococcus aureus* and *Staphylococcus epidermidis* (7,8)) and streptococci (*Streptococcus sanguinis* and *Streptococcus gordonii* (9,10)), very few studies have looked at the mechanisms of interactions between *Escherichia coli and* platelets.

The aim of our study was to investigate platelet aggregation mediated by *Escherichia coli* strains. In this study, ten *Escherichia coli* strains *were* used, five colistin-resistant strains from clinical isolates, and five reference strains, sensitive to colistin, available in the CSUR collection of the IHU Méditerranée Infection. We evaluated the ability of these ten strains to induce platelet aggregation by two complementary methods: spectrophometry and confocal

microscopy. The effect of anti-platelet drugs was evaluated on the strains responsible for platelet aggregation.

Materials and methods

1. Preparation of platelet-rich plasma (PRP)

Blood samples were collected in citrated tubes from healthy volunteers, not taking antibiotics or antiplatelets for 10 days, by venipuncture at the elbow. Platelet-rich plasma (PRP) was obtained by slow centrifugation at 200 rpm for 10 minutes, according to ISTH recommendations (11). Platelet count measurement of PRP was systematically performed using two methods: impedance plus hydroelectric focusing and flow fluorocytometric measurement (Sysmex XN-1000). Patients with platelet counts below 150 G/L in their PRP were excluded. PRPs with platelet counts above 450 G/L were diluted with 0.9% NaCl to bring the platelet concentration down to 400 G/L. After this step, platelet function studies were systematically performed on PRP samples by platelet aggregation in response to several agonists (TRAP, ADP, epinephrine, arachidonic acid), according to international recommendations (11). Our aim was to detect possible drug-induced thrombopathy or platelet pre-activation related to the pre-analytical steps, thus eliminating false activation.

PRP could also be pre-incubated in solutions containing antiplatelet agents: aspirin (Kardegic®, Sanofi-Aventis, France) at a final concentration of 2mM by successive dilutions, ticagrelor (Brilique®, AstraZeneca, Sweden) at a concentration of 10 µM, or a combination of both molecules.

2. Characterisation of *Escherichia coli* strains

The *Escherichia coli* strains used were from the collection of the IHU Méditerranée Infection (Collection des souches de l'unité des Rickettsies, CSUR, Marseille, France). The bacteria were identified by mass spectrometry (MALDI-TOFF, Bruker, Germany). All strains were cryopreserved at -80°C in cryopreservation systems. Ten strains were used from clinical isolates (LH1, LH30, RER60/Q1065, PIA61/Q1066, SEF62/Q6269) or reference strains (ATCC11303, K12, J53, ATCC25922, BL21DE3). Strains were plated on Columbia blood agar enriched with sheep blood (COS, Biomérieux, France) and placed in an oven at 37°C for 24 hours. Solutions were then prepared by diluting colonies from the cultures in 0.9% NaCl.

The target concentrations we used were 10, 12 and 15 McFarland, which were measured using a densitometer (BioSan® DEN-1B). For each strain, the MIC of colistin was determined by microdilution method according to EUCAST recommendations (Table 1).

3. Measurement of platelet aggregation after incubation with *Escherichia coli* strains

Platelet aggregation was measured by spectrophotometric measurement at 740nm on an APACT® 4004 (Elitech, France). 180µ L of platelet-rich plasma (PRP) were mixed with 20 µL of bacteria and agitated at 900 rpm at a temperature of 37°C. Data acquisition was performed for 1200 seconds. Platelet poor plasma (PPP) was also prepared by rapid centrifugation at 1100g for 10 minutes and used to adjust the 100% aggregation. The aggregation capacity of the bacteria was measured by the maximum aggregation percentage, calculated by the following formula: (light transmission before addition of bacteria - light transmission after addition of bacteria) x 100. Two control conditions were systematically performed, one in the presence of 20 µL of 0.9% NaCl, and the other in the presence of 10 µM TRAP (Thrombin Receptor-Activating Peptide, STAGO® , France).

4. Sequencing of strains, *in-silico* serotyping and determination of bacterial virulence genes

For seven of the ten strains used (ATCC25922, ATCC11303, K12, J53, BL21 DE3, LH1 and LH30), the genomes were already available and published on the National Center for Biotechnology Information (NCBI) database. For the other three strains (Q1065, Q1066 and Q6269), pangenome sequencing was performed by Illumina MiSeq pair sequencing. The determination of ORFs (Open Reading Frame) was performed by PROKKA software using the default parameters. The pangenome sequencing data was then processed by Roary software which performed the alignment and comparison of the pangenomes of the different strains.

From the sequencing data obtained, FASTA files were used to determine the serotype of *Escherichia coli* strains *in-silico*. Two software programs were used to ensure concordance of the results. The genomes were first processed by the ECTyper software, available since 2021 (12). The results obtained were then compared to those returned by the SeroTypeFinder module of the Center for Genomic Epidemiology (CGE) (13). The CGE VirulenceFinder module also allowed us to determine *in-silico*, from the bacterial genomes, the different

virulence factors specific to each strain. In order to obtain the mapping of the genes of the different strains, we used the *Genome Browser* function of the EcoCyc database (*ecocyc.org*), which allows us to obtain the relative positions of each of the genes within the *Escherichia coli* genome and to identify the presence of inserted elements within the genome (14).

5. Studies of interactions between *Escherichia coli* strains and platelets by confocal microscopy

After 24 hours of culture on Columbia blood agar enriched with sheep blood (COS, Biomérieux, France), *Escherichia coli* solutions containing a bacterial concentration equal to 2 McFarland were prepared. Two protocols were tested in parallel.

- Protocol 1: Immunostaining on slide. Bacteria were contacted with PRP containing 250 G/L platelets for 220 seconds, which corresponds to the average response time observed in aggregation for strain J53. The mixture was then fixed in 4% paraformaldehyde (PFA) for 15 minutes and rinsed in PBS for 5 minutes. The mixture was then placed on a glass slide to be incubated for 30 minutes with a fluorescent DNA marker for bacterial identification (Hoechst 33342 Solution (20 mM), Thermo Scientific, dil 1:1000), as well as phalloidin for platelet actin labelling (Alexa Fluor™ 488 Phalloidin, Invitrogen, dil 1:500). This labelling step was followed by rinsing in PBS and then distilled water for 5 minutes. The coverslip was then mounted, with the purified water deposit serving as the mounting medium. Reading was performed on a Zeiss LSM800 confocal microscope at different magnifications.
- Protocol 2: Suspension immunostaining. Bacteria were incubated for 30 minutes with a fluorescent DNA marker (Hoechst 33342 (20mM) solution, dil 1:1000). They were then contacted with PRP containing 250 G/L platelets for 220 seconds. The mixture was then fixed in 4% PFA for 15 minutes and rinsed in PBS for 5 minutes. A second marker, phalloidin, for platelet actin labelling (Alexa Fluor™ Phalloidin 488, dil 1:500) was then added to the platelet-bacteria mixture, rinsed for 5 minutes in PBS and then distilled water. The labelled platelet-bacteria mixture was then placed in a Cytospin® device to be spread in a standardised manner on a glass slide (900 rpm for 10 minutes, low acceleration). Finally, the slide mounting step took place, with deposition of a mounting medium containing purified water. The reading was performed on a Zeiss LSM800 confocal microscope at different magnifications.

6. Statistical study

Statistical analysis was performed using GraphPad Prism 9. Statistically significant differences were highlighted using unpaired Student's t-tests with a significance level of $p<0.05$. A normality test was also applied when analysing the effect of antiplatelets. A normality test was also applied when analysing the effect of antiplatelets. The samples followed a Gaussian distribution verified by the Shapiro-Wilk test ($p>0.05$).

Results

1. Identification of platelet aggregation inducing *Escherichia coli* strains

For the ten *Escherichia coli* strains tested, platelet aggregations were performed on 5 PRP samples from different healthy donors. As shown in figure 1A, only 4 strains were able to induce platelet aggregation above 40% at 15 McF (ATCC11303, J53, BL21 DE3, ATCC25922, SEF62). Only the J53 strain induced platelet aggregation above 40% at all three concentrations of *Escherichia coli* tested. In contrast, the effect was dose dependent, with a significant increase in the mean aggregation percentage as the bacterial concentration increased for strains ATCC11303 (19.85%, 10 McF *vs* 45.24%, 15 McF, $p<0.05$) and BL21 DE3 (26.02%, 10 McF *vs* 60.50%, 15 McF, $p<0.05$). In primary analysis, there was a statistically significant difference for strain ATCC11303 at the two lowest concentrations (19.85%, 10 McF *vs.* 34.42%, 12 McF, $p<0.05$), as well as results close to significance for strain BL21 DE3 between 12 McF and 15 McF (41.03%, 12 McF *vs.* 60.50%, 15 McF, $p=0.052$) The colistin-resistant strain SEF62 showed moderate platelet aggregation, exceeding 40% aggregation only at the two highest bacterial concentrations (10 McF: 32.2%, 12 McF: 40.6%, 15 McF: 44.4%, not significant).

Thus, platelet aggregation is observed in both colistin-sensitive and colistin-resistant strains. The ability of strains to aggregate platelets is therefore not dependent on colistin resistance. It is interesting to note that K12, J53 and BL21 DE3 all show different aggregation profiles, despite the fact that the latter are mutant strains derived from the reference strain K12.

2. Determination of virulence factors and in-silico serotyping

In order to identify the mechanisms that could explain the differences in aggregation profile observed between *Escherichia coli* strains, strains were sequenced. The analysis of the results

obtained did not reveal any genes of interest shared by the strains showing similar behaviour (figure 2).

The virulence factors carried by the different strains were determined by *in-silico* sequencing from the FASTA data. Similarly, no common virulence factor for all strains inducing platelet aggregation could be identified by the bioinformatics prediction software, among the 44 virulence factors identified on the 10 *Escherichia coli* strains (figure 3).

However, despite the absence of a common virulence factor to explain the heterogeneity of the results obtained in aggregometry, it was identified in some strains (ATCC11303, ATCC25922, BL21DE3, PIA61/Q1066, SEF62/Q6269) the presence of a gene family called *kps*, which would play a role in the formation of a capsule (K antigen) around the bacterium, thus capable of modifying the interactions between platelet receptors and LPS (table 2, figure 3).

3. O antigen serotyping of *Escherichia coli* strains

Based on these results, we investigated the structures present on the bacterial surface that might be able to modify the interactions between platelets and bacteria, and more particularly LPS. We therefore performed *in-silico* serotyping of the ten strains of interest based on FASTA sequencing data. We were particularly interested in determining the type of O antigen, the outermost part of the LPS, and the H antigen, which characterises the type of flagellum possessed by the bacteria. As shown in Table 3, strains BL21 DE3 and ATCC11303 have an O7 antigen and show relatively similar platelet aggregation profiles. The SEF62/Q6269 strain, which also shows significant platelet aggregation (Figure 1A), expresses a different O serotype to the previous two strains. Strains LH1, LH30, RER60/Q1065 and PIA61/Q1066, which do not induce platelet aggregation, all express different O serotypes (Table 3). Strains J53 and K12 share the same O16:H48 serotype, despite the fact that they have opposite abilities to induce platelet aggregation: strain J53 induces strong platelet aggregation, which is not found with strain K12.

The difference in behaviour between these two strains, which are genetically close as one is derived from the other, was further explored. The GenomeMapper of the EcoCyc database (*ecocyc.org*) allowed us to search for the genes involved in the biosynthesis of the O antigen and LPS. We found that the *wbbL* gene, predicted to play an important role in the biosynthesis of the N-terminal fragment of LPS, had a mobile element called IS5 inserted in its sequence, rendering it non-functional (figure 4). This strain is therefore unable to synthesise an O-

antigen, and therefore shows changes in the structure of LPS that cannot be predicted *in-silico*. From these observations, our secondary hypothesis is that the ability of a strain to induce platelet aggregation is therefore dependent on the type of O antigen present in the LPS of each bacterium.

4. Study of platelet-bacteria interaction by confocal microscopy and determination of signalling pathways

a - Validation of immunostaining protocols

The two immunolabelling protocols (solution labelling and deposition by Cytospin® system *vs*. slide labelling and manual deposition on a glass slide) were compared to determine which one provided the best characterisation of platelet-bacteria interactions. Both protocols resulted in satisfactory platelet labelling, with complete labelling of the actin cytoskeleton, indicating good intra-platelet penetration of the marker (Figure 5). Bacteria were also satisfactorily labelled.

However, the images obtained by the two protocols showed significant differences. Labelling on the slide allows the observation of spontaneous colocalisation between platelets and bacteria, illustrating their ability to interact and induce platelet activation. However, due to the low number of platelets and bacteria present on the slide, this type of labelling does not allow the analysis of the structure of the aggregates obtained with certain strains.

Solution labelling and deposition using the Cytospin® system allows all the elements to be concentrated on a smaller surface. This technique allows the observation of a larger number of elements of interest, but loses the ability to conclude passive colocalisation of bacteria and platelets. Platelet morphology is also less accurate and may be altered during centrifugation. However, analysis of aggregate structure is still possible.

b - Determination of interaction profiles by confocal microscopy

Several parameters were studied in order to determine the interaction profile of each strain with platelets. For each of the strains, the following was investigated:

- The presence of free *Escherichia coli* bacilli on the slide
- The existence of activated platelet morphology in platelet-bacterial colocalisation images

- The presence of aggregates containing both platelets and bacteria, and if present, the location of the bacteria within the aggregate

The presence of colocalisation could indicate the presence of an interaction between these two elements. Colocalisation was defined in our case as the superposition between two elements with different labelling, possibly within a cluster of platelets (Figures 5 and 6). We therefore performed a semi-quantitative approach by looking for the presence of colocalisation images between the two markings, indicating a direct interaction between platelets and bacteria.
Numerous aggregates containing bacilli were observed with strains ATCC11303 and BL21DE3. These observations confirm the results obtained by spectrophometry. Although strain J53 induced a very significant aggregation, the microscopy images showed a lower number of aggregates than those observed for the previously mentioned strains. In addition, the number of bacilli observed is also smaller. Strains ATCC25922 and K12 do not induce platelet aggregates as observed by spectrophotometry. Results are pending for strains PIA61/Q1066, RER60/Q1065 and SEF62/Q6269. All results are summarised in Table 4.

5. Study of the effect of antiplatelets and aggregation mechanisms

The effect of antiplatelet agents was studied on the J53 strain inducing the most platelet aggregation. The effect of two antiplatelet agents was analysed: aspirin, an inhibitor of thromboxane A_2 synthesis, and ticagrelor, an inhibitor of the P_2Y_{12} receptor to ADP.
Pre-treatment of platelets with aspirin (2mM) statistically significantly decreases platelet aggregation regardless of the concentration of J53 strain used (10 McF: 85.09% *vs* 40.38%, $p<0.05$; 12 McF: 87.07% *vs* 61.38%, $p<0.05$). Similar results were obtained with ticagrelor (10 McF: 85.09% *vs* 14.67%, $p<0.05$; 12 McF: 87.07% *vs* 39.10%, $p<0.05$). Although not significant, the decrease was greater with ticagrelor than with aspirin (figure 7).
However, treatment of platelets with an antiplatelet agent does not completely suppress the ability of platelets to aggregate. It should be noted that the reduction in aggregation under antiplatelet therapy appears to be inversely proportional to the bacterial concentration, but not statistically significant (Aspirin: 40.38% (10 McF) *vs* 61.38% (12 McF), $p=0.17$; Ticagrelor: 14.67% (10 McF) vs 39.10% (12 McF), $p=0.13$).

The effect of dual therapy with aspirin and ticagrelor was investigated to determine whether a synergistic action was observed compared to the use of ticagrelor alone. Surprisingly, the use of dual therapy did not significantly reduce platelet aggregation in J53

(10 McF: 23.68% *vs* 23.40%; 12 McF: 42.37% *vs* 38.06%) compared to ticagrelor alone (figure 7). Thus, platelet aggregation persists regardless of the antiplatelet treatment used, with greater aggregation as the bacterial concentration increases.

Discussion

Our results therefore show that aggregation induced by *Escherichia coli* is strain-dependent but also concentration-dependent. This variability observed in aggregometry is found in confocal microscopy. Some strains are able to induce the formation of large platelet aggregates in which the bacilli are found. With other strains, the platelets will remain in a non-activated state despite the proximity to the bacteria. These differences in behaviour are most likely related to differences in the structure of the LPS of the bacteria, and in particular the O antigen.

Watson *et al.* demonstrate in a small number of *Escherichia coli* strains that platelet aggregation induced by two strains, CFT073 and RS218, is strain dependent, one strain being able to induce rapid and potent aggregation, while the other will have no effect, and also concentration dependent, with the platelet/bacteria ratio playing a role in the maximum percentage of aggregation achieved (15).

However, the initial assumption that there was a link between colistin resistance of a bacterial strain and its ability to induce platelet aggregation was thus invalidated, as we observed platelet aggregation with both colistin-susceptible and colistin-resistant strains (Figure 1A).

The analysis of the different pangenomes allowed us to determine whether certain strains possessed genes that could code for proteins capable of inducing platelet activation. Some are already known for other bacterial species, such as clumping factor A (*clfA*) from *Staphylococcus aureus* (16) capable of binding fibrinogen and inducing platelet aggregation. However, we did not identify any virulence factors that could explain the differences observed (Table 2). **Based on the study of virulence factors and serotypes of the different strains used, as well as the aggregometry and microscopy results, we hypothesise that the interaction between platelets and bacteria depends on the structure of the LPS and in particular on the O antigen present on its surface.**

The serotyping results also drew attention to two strains that are genetically very similar: the K12 strain and one of its derived mutant strains, J53, which have opposite properties on platelet aggregation. As previously demonstrated, strain K12 is unable to present a functional O antigen, due to the insertion of a mobile element, IS5, in one of the genes involved in O antigen biosynthesis, the *wbbL* gene (Figure 4) (17–19). From this reference K12 strain

(substrain MG1655), a mutant K12 strain (substrain W3110) was developed by transposing a gene cluster called *rfb*, to which the *wbbL* gene belongs. It was shown that this mutant K12 strain W3110 is capable of expressing a functional O antigen, serotype O:16 (20). Strain J53 is also derived from the reference K12 strain, but spontaneously has a deletion of the mobile element IS5 in the *wbbL* gene, allowing it to express a functional O (21). This difference in LPS structure between two strains with opposite behaviour is the basis for our hypothesis that the serotype of the strains and more specifically the O antigen plays an indispensable role in the interaction with platelets. This hypothesis is also reinforced by the fact that it has been shown that platelets are able to express TLR-4 on their surface, which is involved in the recognition of LPS (22) recognition, and that this TLR-4-mediated signalling pathway is capable of inducing platelet aggregation (23).

Several interaction profiles between platelets and bacteria can therefore be determined, based on the results obtained not only by aggregometry but also by confocal microscopy. These interaction profiles would depend, as mentioned before, on the structure of the LPS and more precisely on the type of O antigen present on the surface of gram-negative bacilli. Indeed, any modification of the LPS structure could therefore lead to a loss of recognition of the bacterial outer membrane by this TLR-4, giving rise to heterogeneous platelet responses as we have observed here. The establishment of these aggregation profiles of the different strains could play an important role in the prognosis of the patient, making it possible to identify the strains most at risk of inducing platelet activation and therefore the patients with an increased risk of developing a thrombotic event. This work should be carried out in parallel with the determination of the serotypes of the *Escherichia coli* strains most frequently involved in bacteremia and sepsis, data for which some recent retrospective studies have already been carried out, which demonstrate that certain serotypes are frequently isolated, in particular serotype O:25 (24,25). These articles echo a previous publication, which already in 2002 demonstrated the capacity of certain LPS with particular serotypes to induce a more important transformation into septic shock than others, when these LPS were injected into mice, and could thus lead to a higher mortality rate (26).

Knowledge of these aggregation profiles could play an important role in determining which serotypes are at greatest risk of developing a thrombotic event, where the use of an antiplatelet agent may be of interest. As we have observed, the effect of ticagrelor appears to be superior to that of aspirin. The combination of these two molecules does not provide a synergistic effect, and would not be of benefit compared to the use of ticagrelor alone. However, the use of antiplatelets, despite a significant decrease in the percentage of maximal

aggregation, does not completely eliminate the ability of platelets to aggregate in the presence of certain strains, notably J53. This persistence of aggregation could be due to the mechanism of activation of platelets by bacteria, which would call upon non-classical signalling pathways. Indeed, it has been shown that TLR-4 is able to recognise LPS from different strains in a heterogeneous manner. Some LPS would bind exclusively to TLR-4, while others would be able to induce the recruitment from plasma of soluble CD14 (sCD14) and induce the formation of a TLR-4-sCD14-LPS complex, involving specific signalling pathways leading to the release of soluble CD40L (sCD40L) (27,28). Soluble CD40L (sCD40L), released from platelet granules in response to this stimulation by the TLR-4-sCD14-LPS (28) complex, is able to induce an increase in the secretion of thromboxane A_2 (29) and potentiate the ability of platelets to aggregate by binding to integrin αIIbβ3 (30)integrin, explaining the persistence of platelet aggregation that bypasses the classical aggregation pathways targeted by aspirin and P_2Y_{12} inhibitors. When looking at CD40L levels in patients treated with different antiplatelet agents, CD40L levels are indeed not significantly altered when taking aspirin, but are lowered when taking a P_2Y_{12} inhibitor (31). This difference could be the reason for our results, explaining both why ticagrelor provides a better reduction in platelet aggregation and the failure of dual therapy with aspirin and ticagrelor compared to ticagrelor alone.

Conclusion

Our work shows that the platelet aggregation profile induced by *Escherichia coli* strains is independent of the colistin resistance state but is dependent on the structure of the LPS. Antiplatelet drugs allow the observed platelet aggregation induced by certain strains to be reduced without completely inhibiting it. In order to better understand these mechanisms, we propose to pursue this work through several complementary approaches:

- the study of the structure of the membrane of bacteria by electronic microscopy
- O antigen detection by microscopy (using a fluorescent O antigen marker)
- the study of signalling pathways leading to activation and therefore to platelet aggregation, in particular the Akt pathway.

In perspective, our objective is to characterise in a prospective study the *Escherichia coli* strains identified in bacteremia and to correlate their profile with the clinical outcome of the patients.

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Figure legends

Figure 1 - A: Platelet aggregation induced by *Escherichia coli* strains at 10 McF (black), 12 McF (grey) or 15 McF (white). N = 5 for each strain and bacterial concentration. *: $p<0.05$ in adjusted analysis; **: $p<0.05$ in primary analysis.

B: Example of aggregation results obtained with strains LH30 and BL21 DE3. The BL21 DE3 strain induces moderate platelet aggregation at all three concentrations, after a lag time of approximately 400 seconds. TRAP is used as a positive control and spontaneous aggregation as a negative control. 1

Figure 2- Comparison of different pangenomes obtained by NGS sequencing. All ten strains show a low level of clusterisation.

Figure 3*: Heatmap of* the 44 virulence factors identified in the different *Escherichia coli* strains. No common virulence factors inducing platelet aggregation could be identified for all strains

Figure 4*:* Comparison of the structure and biosynthesis of the O antigen of strains K12 and J53. Strain K12 does not have a functional O antigen, secondary to the presence of a mobile element, IS5, inserted into the *wbbL* gene involved in O antigen synthesis.

Figure 5*:* Images of colocalisation (A, C) or lack of colocalisation (B) between the two labels (x63 objective, mosaic images). The presence of bacteria within a smooth looking aggregate is noted for the BL21 DE3 strain (C) (x63 immersion lens). The colocalisation results observed seem to be consistent with the results found in aggregometry.

Figure 6: Confocal microscopy images of aggregates with a very heterogeneous appearance depending on the strain (x63 immersion objective).
A and B: Absence of platelet aggregates in response to strain ATCC 25922.
C: Presence of numerous small aggregates in response to J53. There is a change in the general appearance of the platelets in response to this strain.

D and E: Presence of large aggregates in response to ATCC 11303 (Z-stack) and BL21 DE3 (3D rendering, obtained by orthogonal projection). Some bacilli are visible in the middle of the aggregate, but also outside.

F: Presence of large aggregates in response to strain LH30. No bacteria are visible outside these structures.

Figure 7: Effect of antiplatelet agents on aggregation induced by *Escherichia coli* strain J53 in the presence of aspirin (white), ticagrelor (grey), dual therapy with aspirin + ticagrelor (barred) or without antiplatelet therapy (black). Statistical significance: * = $p<0.05$ in adjusted analysis, ** = $p<0.05$ in primary analysis. N = 5 for each condition

Table legends

Table 1- Origin and sensitivity of the *Escherichia coli* strains used.

MIC = minimum inhibitory concentration

Table 2: Example of in-silico prediction result for virulence factors of Escherichia coli SEF62. The function of each gene is also predicted.

Table 3: Results of serotyping data using the ECTyper in-silico prediction program, allowing determination of the O and H antigens carried by each Escherichia coli strain

Table 4: Summary table of the results obtained by in-silico serotyping, aggregometry and microscopy with the 10 Escherichia coli strains. Two strains with the same serotype appear to have similar profiles. EC: In progress

Figure 1

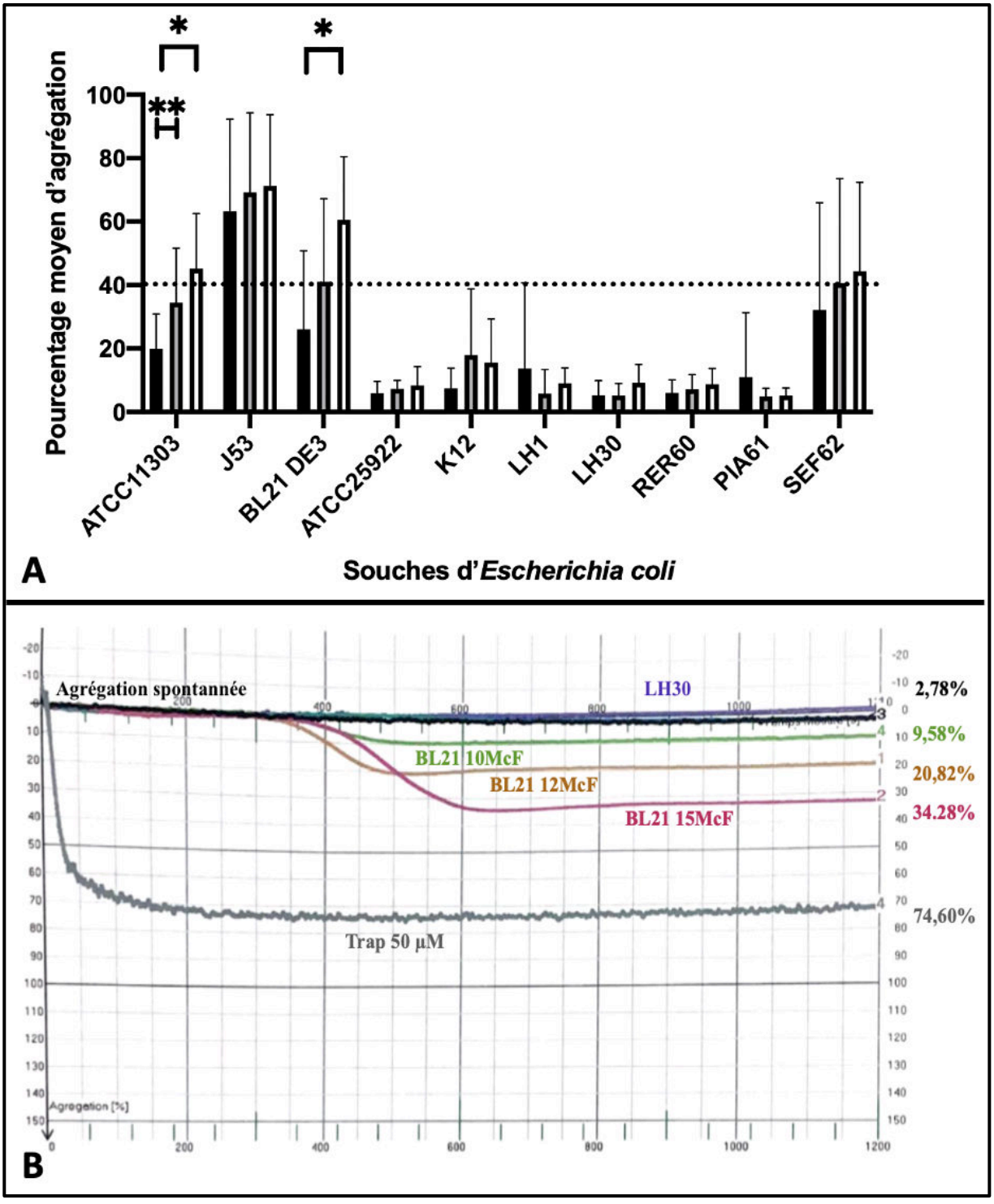
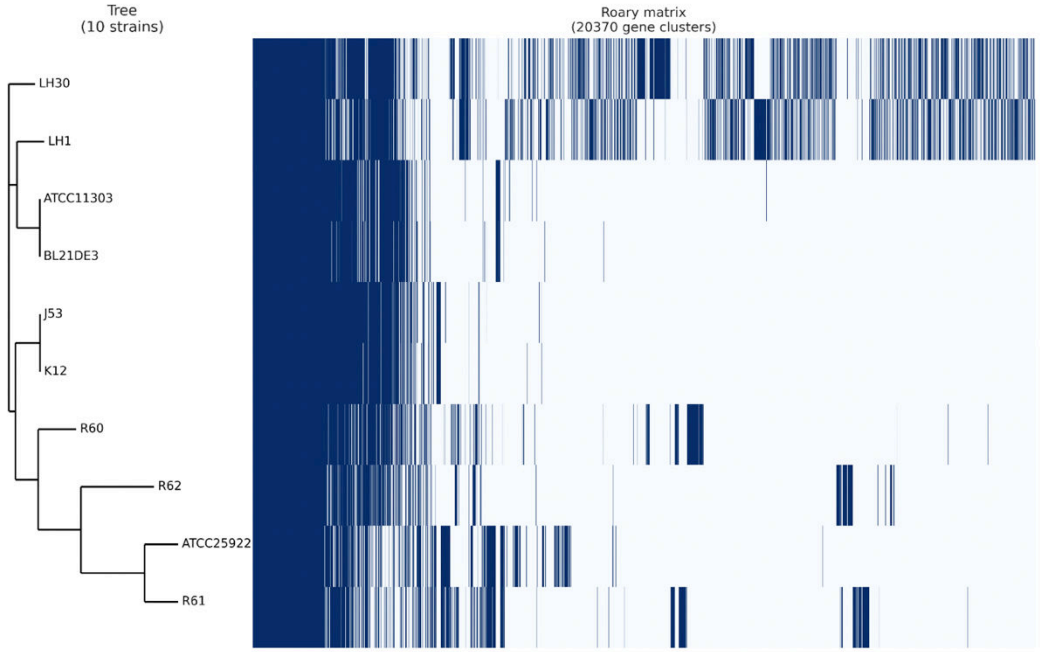

Pourcentage moyen d'agrégation
100
80
60
40
20
0
*
*
**
ATCC11303
J53
BL21 DE3
ATCC25922
K12
LH1
LH30
RER60
PIA61
SEF62
A
Souches d'Escherichia coli
Agrégation spontannée
LH30
2,78%
BL21 10McF
9,58%
BL21 12McF
20,82%
BL21 15McF
34.28%
Trap 50 µM
74,60%
Agregation [%]
B

Figure 2:


Tree
(10 strains)
Roary matrix
(20370 gene clusters)
LH30
LH1
ATCC11303
BL21DE3
J53
K12
R60
R62
ATCC25922
R61

462 **Figure 3:**

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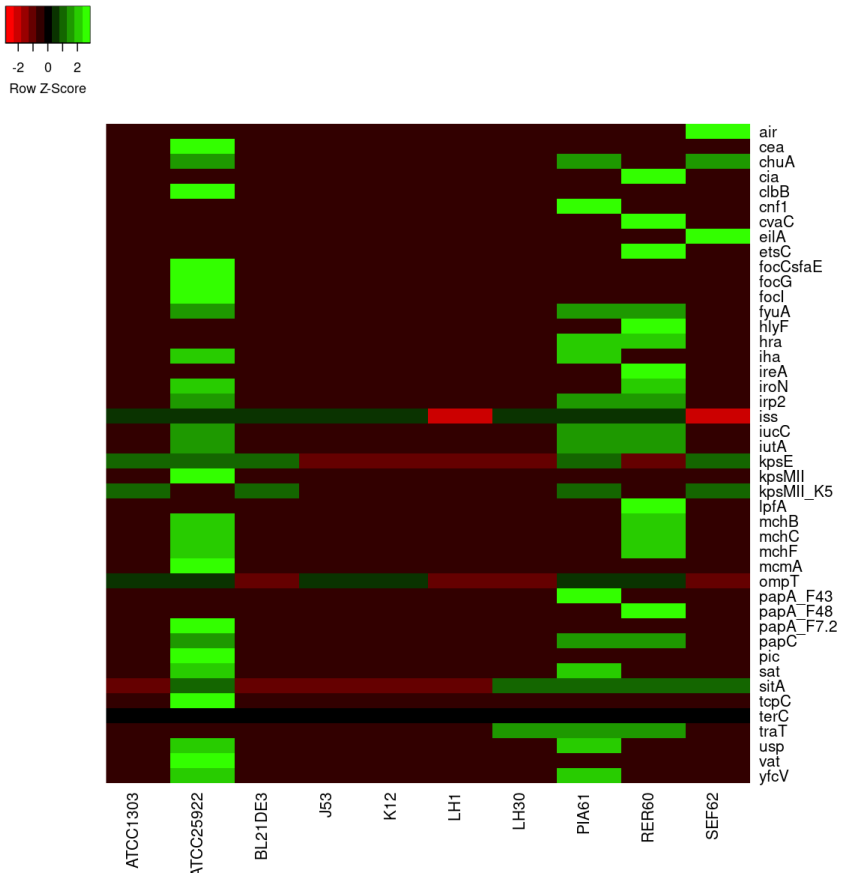

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Figure 4

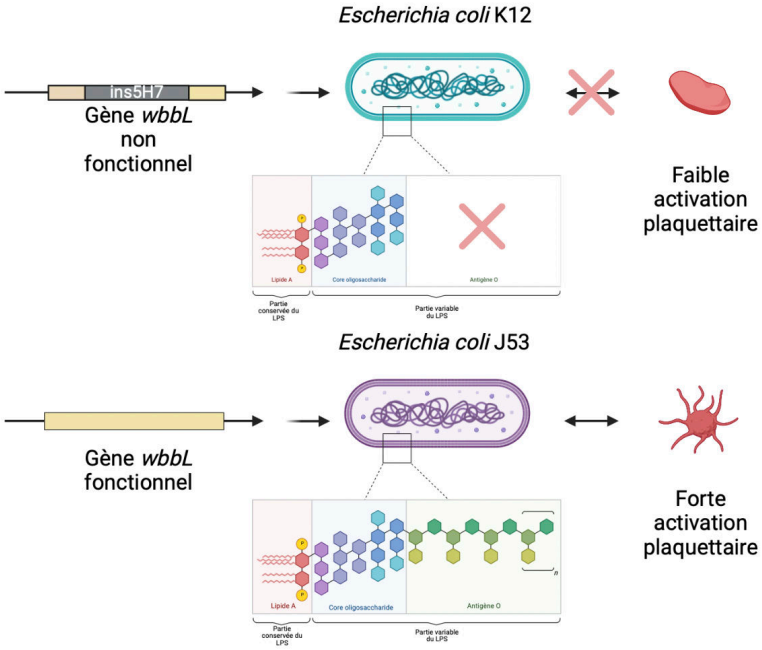

Escherichia coli K12
ins5H7
Gène *wbbL*
non
fonctionnel
Faible
activation
plaquettaire
Escherichia coli J53
Gène *wbbL*
fonctionnel
Forte
activation
plaquettaire

Figure 5

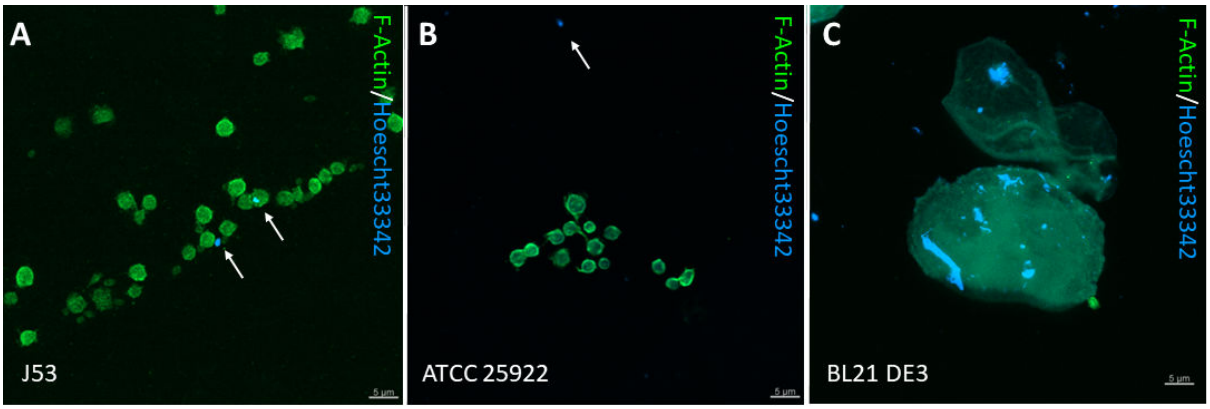
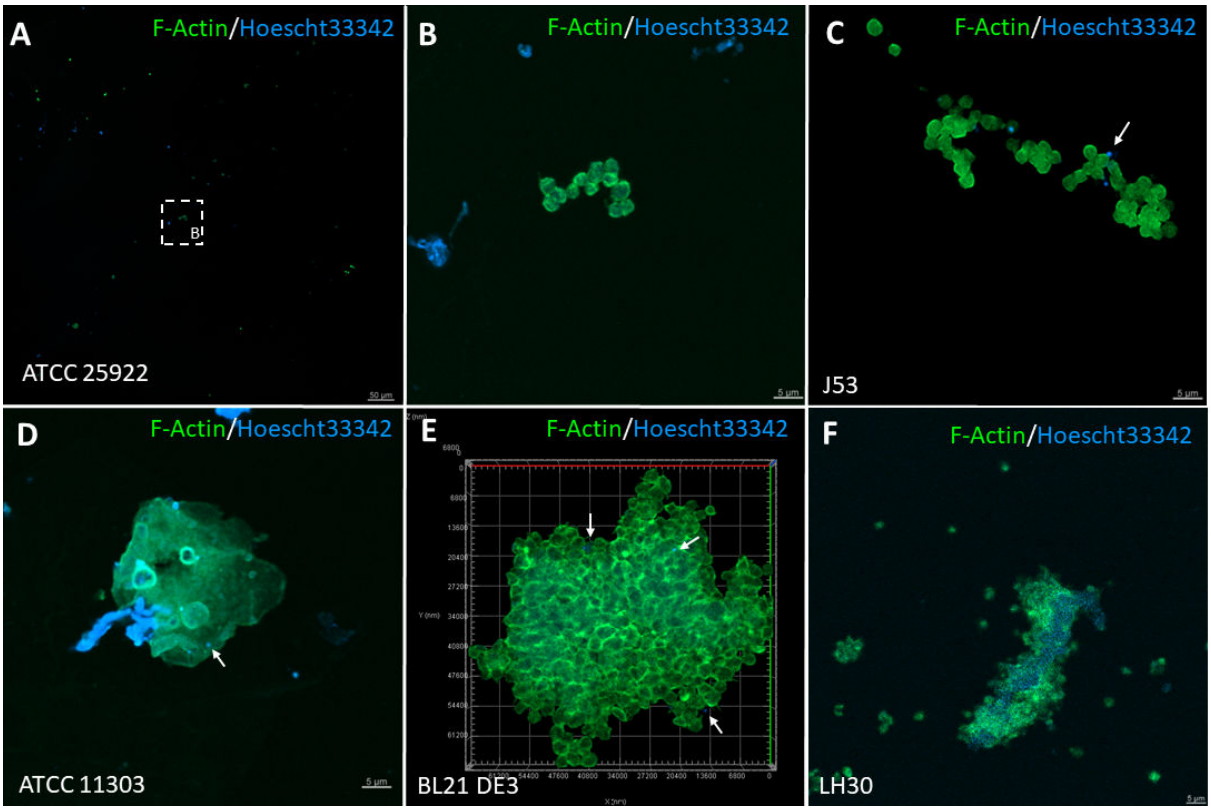

A
F-Actin/Hoescht33342
J53
B
F-Actin/Hoescht33342
ATCC 25922
C
F-Actin/Hoescht33342
BL21 DE3

Figure 6:


A
F-Actin/Hoescht33342
ATCC 25922
B
F-Actin/Hoescht33342
C
F-Actin/Hoescht33342
J53
D
F-Actin/Hoescht33342
ATCC 11303
E
F-Actin/Hoescht33342
BL21 DE3
F
F-Actin/Hoescht33342
LH30

484 **Figure 7:**

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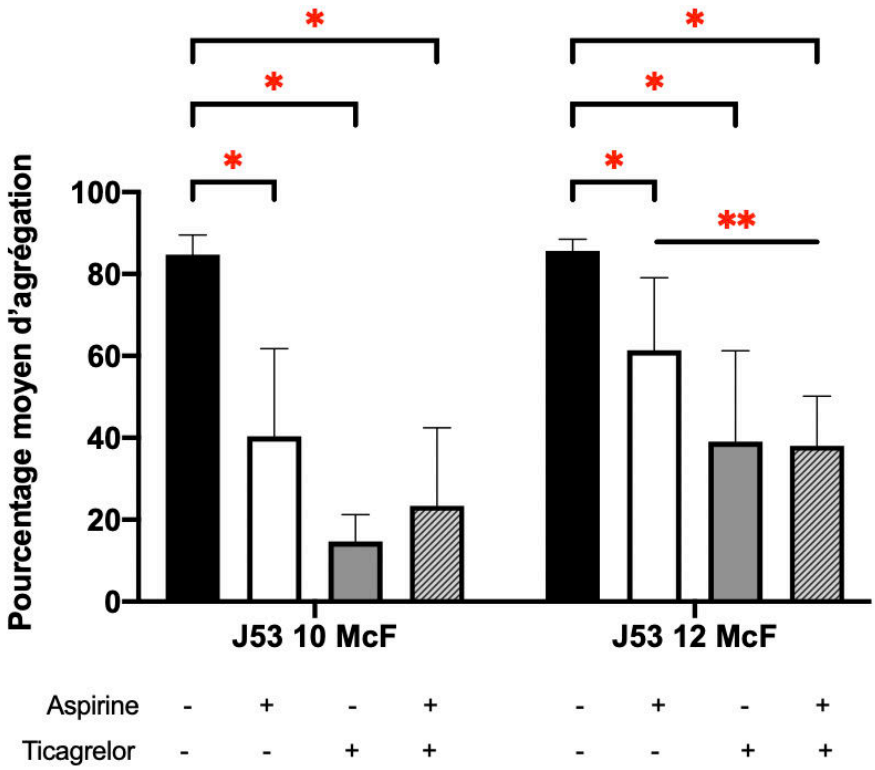

487

488

Table 1

Souches d'*Escherichia coli*	Origine	CMI colistine (mg/L) (32)	Sensibilité à la colistine	Mécanisme de résistance (32)
LH1	Humaine	7,8	Résistant	Gène *mcr-1*
LH30	Humaine	3,9	Résistant	Gène *mcr-1*
PIA61/Q1066	Humaine (prélèvement rectal)	7,8	Résistant	Mécanisme inconnu
RER60/Q1065	Humaine (prélèvement pharyngé)	3,9	Résistant	Mécanisme inconnu
SEF62/Q6269	Humaine (urine)	3,9	Résistant	Mécanisme inconnu
ATCC25922	Souche de référence	0,97	Sensible	
ATCC11303	Souche de référence	0,48	Sensible	
K12	Souche de référence	1,95	Sensible	
J53	Souche de référence (mutant de la K12)	0,97	Sensible	
BL21 DE3	Souche de référence (mutant de la K12)	0,97	Sensible	

494 **Table 2**

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Virulence_SEF62

Database	Virulence factor	Identity	Query / Template length	Contig	Position in contig	Protein function	Accession number
virulence_ecoli	air	95.48	4606 / 4605	NODE_8_length_225858_cov_19.9126	115175..119780	Enteroaggregative immunoglobulin repeat protein	CP003034
virulence_ecoli	chuA	99.95	1983 / 1983	NODE_10_length_197674_cov_19.6138	178465..180447	Outer membrane hemin receptor	UGFM01000001
virulence_ecoli	eilA	99.18	1698 / 1698	NODE_8_length_225858_cov_19.9126	127797..129494	Salmonella HilA homolog	FN554766
virulence_ecoli	gad	98.86	875 / 1401	NODE_52_length_875_cov_28.9318	1..875	Glutamate decarboxylase	CU928162
virulence_ecoli	kpsE	99.83	1149 / 1149	NODE_1_length_506624_cov_19.0891	155161..156309	Capsule polysaccharide export inner-membrane protein	AM946981
virulence_ecoli	kpsMII_K5	100.0	777 / 777	NODE_1_length_506624_cov_19.0891	142545..143321	Polysialic acid transport protein; Group 2 capsule	UGCH01000001
virulence_ecoli	sitA	100.0	915 / 915	NODE_2_length_323237_cov_17.9383	240071..240985	Iron transport protein	ADTM01000098
virulence_ecoli	terC	99.02	714 / 714	NODE_1_length_506624_cov_19.0891	321269..321982	Tellurium ion resistance protein	CP000468
virulence_ecoli	terC	98.54	959 / 966	NODE_11_length_180785_cov_19.0791	6481..7439	Tellurium ion resistance protein	MG591698

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498 **Table 3**

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Escherichia coli strains	O-type	H-type	Serotype	GeneCoverages(%)	Agrégation
BL21DE3	O7	-	O7:-	100;100;	+
Escherichia_coli_ATCC_11303	O7	-	O7:-	100;100;	+
Escherichia_coli_ATCC_25922	O6	H1	O6:H1	100;100;100;	-
J53	O16	H48	O16:H48	100;100;100;	+++
K12MG1655	O16	H48	O16:H48	100;100;100;	-
LH1	O174	H2	O174:H2	100;100;	-
LH30	O8	H4	O8:H4	100;100;100;	-
RER60/Q1065	O9	H25	O9:H25	100;100;100;	-
PIA61/Q1066	O25	H4	O25:H4	100;100;100;	-
SEF62/Q6269	O175	H15	O175:H15	100;100;	+

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502 **Table 4**

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Souche d'*Escherichia coli*	Sérotype	Agrégomètrie	Bacilles libres visibles en microscopie	Agrégats plaquettaires en microscopie	Aspect de plaquettes activées	Bactéries dans les agrégats
LH1	O174:H2	-	++	+/-	-	+/-
LH30	O8:H4	-	-	++	+/-	++
PIA61/Q1066	O25:H4	-	EC	EC	EC	EC
RER60/Q1065	O9:H25	-	EC	EC	EC	EC
SEF62/Q6269	O175:H15	+	EC	EC	EC	EC
ATCC25922	O6:H1	-	++	-	+/-	-
K12	- :H48	-	+	-	+/-	-
J53	O16:H48	++	+	+/-	?	+
ATCC11303	O7:-	+	++	+	++	++
BL21 DE3	O7:-	+	++	+	++	++

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References

1. Vallance TM, Zeuner MT, Williams HF, Widera D, Vaiyapuri S. Toll-Like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis. Mediators Inflamm. 2017;2017:9605894.

2. Armant MA, Fenton MJ. Toll-like receptors: a family of pattern-recognition receptors in mammals. Genome Biology. 29 juill 2002;3(8):reviews3011.1.

3. Kaper JB, Nataro JP, Mobley HL. Pathogenic Escherichia coli. Nat Rev Microbiol. févr 2004;2(2):123-40.

4. Lüthje P, Brauner A. Virulence factors of uropathogenic E. coli and their interaction with the host. Adv Microb Physiol. 2014;65:337-72.

5. Poirel L, Madec JY, Lupo A, Schink AK, Kieffer N, Nordmann P, et al. Antimicrobial Resistance in Escherichia coli. Microbiol Spectr. juill 2018;6(4).

6. Ling Z, Yin W, Shen Z, Wang Y, Shen J, Walsh TR. Epidemiology of mobile colistin resistance genes mcr-1 to mcr-9. Journal of Antimicrobial Chemotherapy. 1 nov 2020;75(11):3087-95.

7. Herrmann M, Lai QJ, Albrecht RM, Mosher DF, Proctor RA. Adhesion of Staphylococcus aureus to surface-bound platelets: role of fibrinogen/fibrin and platelet integrins. J Infect Dis. févr 1993;167(2):312-22.

8. Bayer AS, Sullam PM, Ramos M, Li C, Cheung AL, Yeaman MR. Staphylococcus aureus induces platelet aggregation via a fibrinogen-dependent mechanism which is independent of principal platelet glycoprotein IIb/IIIa fibrinogen-binding domains. Infect Immun. sept 1995;63(9):3634-41.

9. Kurpiewski GE, Forrester LJ, Campbell BJ, Barrett JT. Platelet aggregation by Streptococcus pyogenes. Infect Immun. févr 1983;39(2):704-8.

10. Keane C, Tilley D, Cunningham A, Smolenski A, Kadioglu A, Cox D, et al. Invasive Streptococcus pneumoniae trigger platelet activation via Toll-like receptor 2. J Thromb Haemost. déc 2010;8(12):2757-65.

11. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost. juin 2013;11(6):1183-9.

12. Bessonov K, Laing C, Robertson J, Yong I, Ziebell K, Gannon VPJ, et al. ECTyper: in silico Escherichia coli serotype and species prediction from raw and assembled whole-genome sequence data. Microbial Genomics [Internet]. 3 déc 2021 [cité 1 juill 2022];7(12). Disponible sur: https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000728

13. Joensen KG, Tetzschner AMM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and Easy *In Silico* Serotyping of Escherichia coli Isolates by Use of Whole-Genome Sequencing Data. Carroll KC, éditeur. J Clin Microbiol. août 2015;53(8):2410-26.

14. Keseler IM, Gama-Castro S, Mackie A, Billington R, Bonavides-Martínez C, Caspi R, et al. The EcoCyc Database in 2021. Front Microbiol. 2021;12:711077.

15. Watson CN, Kerrigan SW, Cox D, Henderson IR, Watson SP, Arman M. Human platelet activation by Escherichia coli: roles for FcγRIIA and integrin αIIbβ3. Platelets. sept 2016;27(6):535-40.

16. O´Brien L, Kerrigan SW, Kaw G, Hogan M, Penadés J, Litt D, et al. Multiple mechanisms for the activation of human platelet aggregation by Staphylococcus aureus: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A: Multiple mechanisms for stimulating platelet aggregation in S. aureus. Molecular Microbiology. 9 mai 2002;44(4):1033-44.

17. Jeong H, Barbe V, Lee CH, Vallenet D, Yu DS, Choi SH, et al. Genome Sequences of Escherichia coli B strains REL606 and BL21(DE3). Journal of Molecular Biology. déc 2009;394(4):644-52.

18. Matsumura Y, Peirano G, Pitout JDD. Complete Genome Sequence of Escherichia coli J53, an Azide-Resistant Laboratory Strain Used for Conjugation Experiments. Genome Announc. 24 mai 2018;6(21):e00433-18.

19. Stevenson G, Neal B, Liu D, Hobbs M, Packer NH, Batley M, et al. Structure of the O antigen of Escherichia coli K-12 and the sequence of its rfb gene cluster. J Bacteriol. juill 1994;176(13):4144-56.

20. Bilge SS, Vary JC, Dowell SF, Tarr PI. Role of the Escherichia coli O157:H7 O side chain in adherence and analysis of an rfb locus. Infect Immun. nov 1996;64(11):4795-801.

21. Matsumura Y, Peirano G, Pitout JDD. Complete Genome Sequence of Escherichia coli J53, an Azide-Resistant Laboratory Strain Used for Conjugation Experiments. Genome Announc. 24 mai 2018;6(21):e00433-18.

22. Andonegui G, Kerfoot SM, McNagny K, Ebbert KVJ, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. Blood. 1 oct 2005;106(7):2417-23.

23. Niklaus M, Klingler P, Weber K, Koessler A, Boeck M, Kobsar A, et al. The involvement of toll-like receptors 2 and 4 in human platelet signalling pathways. Cell Signal. déc 2020;76:109817.

24. Verboom DM, Varkila MRJ, Morrow B, Davies T, Ibarra de Palacios P, Poolman J, et al. O-serotype distribution of Escherichia coli bloodstream infection isolates in critically ill patients in The Netherlands. Vaccine. mars 2021;39(12):1670-4.

25. Paramita RI, Nelwan EJ, Fadilah F, Renesteen E, Puspandari N, Erlina L. Genome-based characterization of Escherichia coli causing bloodstream infection through next-generation sequencing. Karunasagar I, éditeur. PLoS ONE. 23 déc 2020;15(12):e0244358.

582 26. Zhao L, Ohtaki Y, Yamaguchi K, Matsushita M, Fujita T, Yokochi T, et al. LPS-induced
583 platelet response and rapid shock in mice: contribution of O-antigen region of LPS and
584 involvement of the lectin pathway of the complement system. Blood. 1 nov
585 2002;100(9):3233-9.

586 27. Berthet J, Damien P, Hamzeh-Cognasse H, Arthaud CA, Eyraud MA, Zéni F, et al.
587 Human platelets can discriminate between various bacterial LPS isoforms via TLR4
588 signaling and differential cytokine secretion. Clinical Immunology. déc
589 2012;145(3):189-200.

590 28. Damien P, Cognasse F, Eyraud MA, Arthaud CA, Pozzetto B, Garraud O, et al. LPS
591 stimulation of purified human platelets is partly dependent on plasma soluble CD14 to
592 secrete their main secreted product, soluble-CD40-Ligand. BMC Immunol. déc
593 2015;16(1):3.

594 29. Kojok K, Mohsen M, El Kadiry AEH, Mourad W, Merhi Y. Aspirin Reduces the
595 Potentiating Effect of CD40L on Platelet Aggregation via Inhibition of Myosin Light
596 Chain. JAHA. 4 févr 2020;9(3):e013396.

597 30. Davì G, Ferroni P. CD40-CD40L interactions in platelet activation. Thromb Haemost.
598 2005;93(06):1011-2.

599 31. Judge HM, Buckland RJ, Holgate CE, Storey RF. Glycoprotein IIb/IIIa and P2Y $_{12}$
600 receptor antagonists yield additive inhibition of platelet aggregation, granule secretion,
601 soluble CD40L release and procoagulant responses. Platelets. nov 2005;16(7):398-407.

602 32. Ezzeroug Ezzraimi A, Hannachi N, Mariotti A, Rolland C, Levasseur A, Baron SA, et al.
603 The Antibacterial Effect of Platelets on Escherichia coli Strains. Biomedicines. 28 juin
604 2022;10(7):1533.

605

Conclusion et perspectives

Les travaux présentés dans cette thèse ont pour objectif de participer à la compréhension des mécanismes d'interaction entre les plaquettes et *E. coli* et à identifier les conséquences de cette interaction sur les plaquettes, mais aussi sur les bactéries.

Les conclusions des travaux précédemment disponibles dans la littérature sur ce thème reposent classiquement sur un nombre restreint de souches. Notre analyse bibliographique nous a permis de démontrer que les conclusions obtenues étaient souvent contradictoires. Ce travail inaugural nous a aidé à établir notre modèle d'étude. Nous avons systématiquement utilisé un large panel de souches qui étaient soit des souches de laboratoire et soit issues d'isolats cliniques pour mettre en exergues les différences de réponses.

Nous avons démontré que l'interaction entre les plaquettes est *E. coli* est bidirectionnelle. Les bactéries peuvent induire une activation plaquettaire voire une agrégation, et les plaquettes à leur tour relarguent des molécules granulaires qui peuvent tuer les bactéries. En revanche, les conséquences de ces interactions diffèrent entre les souches testées. À notre connaissance, nous sommes les premiers à démontrer une différence de comportement en fonction des souches, en utilisant un nombre de dix souches avec des profils différents. Certaines souches induisent une activation plaquettaire et d'autres non. Ces différences s'illustrent principalement dans la croissance bactérienne. Les résultats du premier travail (article 2) montrent que la diminution de nombre de colonies d'*E. coli* en présence de plaquettes est liée au phénomène d'activation plaquettaire. Plus les bactéries induisent une activation, moins les bactéries sont présentes après leur incubation avec les plaquettes. Ces observations nous ont montré que l'activité bactéricide des plaquettes est d'origine sécrétoire et qu'elle est dépendante du niveau d'activation. La différence de comportement des souches d'*E. coli* vis-à-vis des plaquettes a été analysée par comparaison génomique. Cela ne nous a pas permis d'identifier le gène ou le groupe de gènes originaire de cette différence. Néanmoins, en en se basant sur les données de la littérature, nous nous sommes intéressées à deux souches apparentées mais qui présentent des profils opposés. Nos résultats ont démontré le rôle de l'antigène-O dans ces interactions. En effet, la présence ou l'absence de l'antigène-O explique que le LPS soit de type rugueux ou lisse. Cette différence pourra influencer la réponse des bactéries et des plaquettes lors de leur interaction. Nos résultats démontrent en revanche que ces différences de comportement sont indépendantes de la résistance ou de la sensibilité à la colistine.

L'analyse par SEM des préparations plaquettes-*E. coli* a permis d'expliquer les différences de réponse observées précédemment. Effectivement, l'aspect des préparations plaquettes-*E. coli* est principalement lié au niveau d'activation. Les souches qui n'activent pas les plaquettes se trouvent avec un nombre important côte à côte des plaquettes et celles qui induisent une activation modérée se trouvent mélangés avec les plaquettes. En revanche, les souches qui induisent une forte activation se trouvent piégées à l'intérieur du magma plaquettaire qui est le contenu relargué des granules lors d'une activation. Nous pouvons donc émettre l'hypothèse que certaines souches d'*E. coli* en cas de bactériémie sont détruites par les plaquettes. L'ensemble des résultats de ce premier axe, nous ont apporté un gain considérable d'information sur l'interaction et ses conséquences entre les plaquettes et *E. coli*, à savoir l'activation et la bactéricidie.

Le second axe de notre travail concerne l'agrégation plaquettaire induite par les souches d'*E. coli.* L'évaluation a été faite en utilisant les souches testées précédemment avec des concentrations bactériennes croissantes.

Certaines souches présentent de faibles niveaux d'interaction avec les plaquettes et induisent une faible agrégation plaquettaire. A l'inverse, d'autres souches induisent une forte agrégation plaquettaire. Nos résultats démontrent que l'agrégation est indépendante des profils de résistance à la colistine. Ce phénomène d'agrégation est donc variable et également principalement souche-dépendant. Nous émettons l'hypothèse que la capacité des souches à induire une agrégation est liée aux sérotypes de l'antigène-O et à sa fonctionnalité. Cette hypothèse est appuyée :

- Premièrement, par le fait que certaines souches, qui présentent le même sérotype, induisent le même profil d'agrégation
- Deuxièmement par le fait qu'un même sérotype associé à un antigène-O fonctionnel ou pas présente les profils d'agrégation différents.

Les phénomènes d'agrégation, en situation de bactériémie, peuvent exposer les patients à un phénomène d'immunothrombose. Ainsi, les antiplaquettaires pourraient jouer un rôle important dans la prise en charge du sepsis, et particulièrement à *E. coli*. Cependant, aucune étude clinique n'a évalué le bénéfice des antiplaquettaires dans le sepsis en fonction de l'espèce bactérienne. Dans ce travail, nous avons évalué *in vitro* l'effet de deux molécules antiplaquettaires testées seules et combinées sur l'agrégation induite par une souche de référence. Le traitement des plaquettes par les agents antiplaquettaires, les plus fréquemment prescrits, diminue la capacité des plaquettes à s'agréger sans l'inhiber totalement. De plus, la

bithérapie ne réduit pas l'agrégation de manière significative. Ces résultats suggèrent la mise en œuvre d'autres voies d'activation plaquettaire lors de ces interactions.

Cette thèse a permis pour la première fois d'étudier l'interaction bidirectionnelle entre les plaquettes et un large panel de souches d'*E. coli.* Cependant, nos travaux soulèvent également de nombreuses questions et plusieurs approches complémentaires pourraient être envisagées (figure 2).

Nos résultats démontrent l'activité bactéricide des plaquettes sur certaines souches de *E. coli*, nous proposons de caractériser les PMPs impliquées dans ces phénomènes par spectrométrie de masse dans un premier temps mais également par séquençage. Cela nous permettra de comprendre le mode d'action de ces PMPs et d'évaluer leurs effets sur les souches cliniques.

A propos des mécanismes d'activation et d'agrégation plaquettaire, nous proposons d'étudier :

- Les voies d'activation plaquettaire mises en jeu lors de l'interaction avec les différentes souches, notamment la voie Akt.
- L'action d'autres agents antiplaquettaires notamment les anti-GPIIbIIIa (tirofiban).

Au regard des phénomènes d'immunothromboses décrits lors des bactériémies à *E coli,* les conséquences de ces interactions sur l'activité procoagulante des plaquettes n'ont pas étudiées. Nous proposons de rechercher l'expression de facteur tissulaire (FT) par les plaquettes par immunomarquage mais également de procéder à des tests globaux tels que la génération de thrombine à partir des mélanges plaquettes-*E. coli*.

Enfin, dans le cadre d'une étude clinique prospective chez les patients présentant un sepsis à *E. coli*, il serait utile de caractériser les souches isolées à partir des hémocultures. L'objectif de notre équipe serait de corréler l'évolution des patients aux caractéristiques des souches en réalisant :

- Leur sérotypage, en étudiant notamment l'antigène-O, sa présence et sa fonctionnalité
- Une description de la surface des souches par microscopie électronique
- Leur profil d'agrégation et leur réponse en présence d'agents antiplaquettaires.

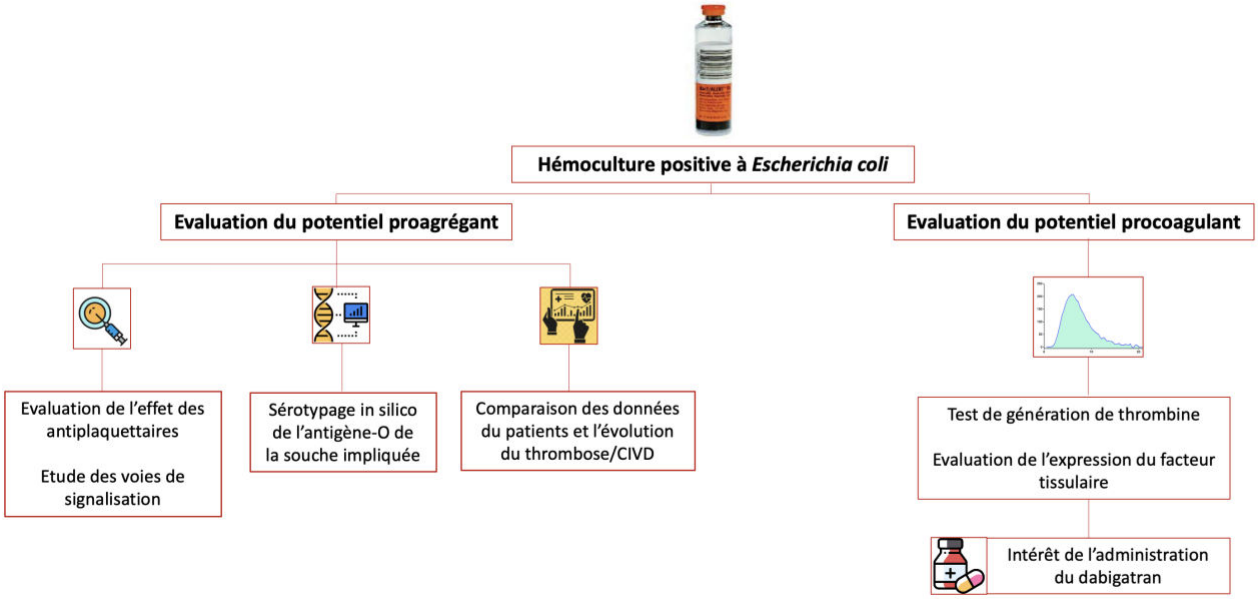

Figure 2 : Schéma récapitulatif des perspectives abordées

Références

1. Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C, Garraud O. Evidence of Toll-like receptor molecules on human platelets. Immunol Cell Biol. avr 2005;83(2):196-8.

2. Fitzgerald JR, Foster TJ, Cox D. The interaction of bacterial pathogens with platelets. Nat Rev Microbiol [Internet]. 1 juin 2006 [cité 20 sept 2022];4(6):445-57. Disponible sur: https://www.nature.com/articles/nrmicro1425

3. Hamzeh-Cognasse H, Damien P, Chabert A, Pozzetto B, Cognasse F, Garraud O. Platelets and infections - complex interactions with bacteria. Front Immunol. 2015;6:82.

4. Koupenova M, Vitseva O, MacKay CR, Beaulieu LM, Benjamin EJ, Mick E, et al. Platelet-TLR7 mediates host survival and platelet count during viral infection in the absence of platelet-dependent thrombosis. Blood. 31 juill 2014;124(5):791-802.

5. Shiraki R, Inoue N, Kawasaki S, Takei A, Kadotani M, Ohnishi Y, et al. Expression of Toll-like receptors on human platelets. Thrombosis Research [Internet]. janv 2004 [cité 28 janv 2022];113(6):379-85. Disponible sur: https://linkinghub.elsevier.com/retrieve/pii/S0049384804001847

6. Yeaman MR. Bacterial-platelet interactions: virulence meets host defense. Future Microbiol. mars 2010;5(3):471-506.

7. Cox D, Kerrigan SW, Watson SP. Platelets and the innate immune system: mechanisms of bacterial-induced platelet activation. J Thromb Haemost. juin 2011;9(6):1097-107.

8. Aquino-Domínguez AS, Romero-Tlalolini M de LA, Torres-Aguilar H, Aguilar-Ruiz SR. Recent Advances in the Discovery and Function of Antimicrobial Molecules in Platelets. Int J Mol Sci. 23 sept 2021;22(19):10230.

9. Krijgsveld J, Zaat SA, Meeldijk J, van Veelen PA, Fang G, Poolman B, et al. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. J Biol Chem. 7 juill 2000;275(27):20374-81.

10. Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. Crit Rev Biotechnol. juin 2012;32(2):143-71.

11. Yeaman MR. Platelets: at the nexus of antimicrobial defence. Nat Rev Microbiol [Internet]. juin 2014 [cité 16 déc 2021];12(6):426-37. Disponible sur: http://www.nature.com/articles/nrmicro3269

12. Watson CN, Kerrigan SW, Cox D, Henderson IR, Watson SP, Arman M. Human platelet activation by Escherichia coli: roles for FcγRIIA and integrin αIIbβ3. Platelets. sept 2016;27(6):535-40.

13. Ezzeroug Ezzraimi A, Hannachi N, Mariotti A, Rolain JM, Camoin-Jau L. Platelets and Escherichia coli: A Complex Interaction. Biomedicines. 7 juill 2022;10(7):1636.

14. Ezzeroug Ezzraimi A, Hannachi N, Mariotti A, Rolland C, Levasseur A, Baron SA, et al. The Antibacterial Effect of Platelets on Escherichia coli Strains. Biomedicines. 28 juin 2022;10(7):1533.

15. Shannon O. Determining Platelet Activation and Aggregation in Response to Bacteria. In: Nordenfelt P, Collin M, éditeurs. Bacterial Pathogenesis [Internet]. New York, NY: Springer New York; 2017 [cité 28 juill 2022]. p. 267-73. (Methods in Molecular Biology; vol. 1535). Disponible sur: http://link.springer.com/10.1007/978-1-4939-6673-8_17

16. Hannachi N, Grac L, Baudoin JP, Fournier PE, Habib G, Camoin-Jau L. Effect of antiplatelet agents on platelet antistaphylococcal capacity: An in vitro study. International Journal of Antimicrobial Agents [Internet]. mars 2020 [cité 16 déc 2021];55(3):105890. Disponible sur: https://linkinghub.elsevier.com/retrieve/pii/S0924857920300297

17. O'Seaghdha M, van Schooten CJ, Kerrigan SW, Emsley J, Silverman GJ, Cox D, et al. Staphylococcus aureus protein A binding to von Willebrand factor A1 domain is mediated by conserved IgG binding regions. FEBS J. nov 2006;273(21):4831-41.

18. Youssefian T, Drouin A, Massé JM, Guichard J, Cramer EM. Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation. Blood. 1 juin 2002;99(11):4021-9.

19. Hannachi N, Fournier PE, Martel H, Habib G, Camoin-Jau L. Statins potentiate the antibacterial effect of platelets on *Staphylococcus aureus*. Platelets [Internet]. 4 juill 2021 [cité 16 déc 2021];32(5):671-6. Disponible sur: https://www.tandfonline.com/doi/full/10.1080/09537104.2020.1792434

20. Kaper JB, Nataro JP, Mobley HL. Pathogenic Escherichia coli. Nat Rev Microbiol. févr 2004;2(2):123-40.

21. Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U, Cantón R, et al. Escherichia coli: an old friend with new tidings. FEMS Microbiol Rev. 1 juill 2016;40(4):437-63.

22. Petruzziello-Pellegrini TN, Moslemi-Naeini M, Marsden PA. New insights into Shiga toxin-mediated endothelial dysfunction in hemolytic uremic syndrome. Virulence. 15 août 2013;4(6):556-63.

23. Matus V, Valenzuela JG, Hidalgo P, Pozo LM, Panes O, Wozniak A, et al. Human platelet interaction with E. coli O111 promotes tissue-factor-dependent procoagulant activity, involving Toll like receptor 4. PLoS One. 2017;12(9):e0185431.

24. Moriarty RD, Cox A, McCall M, Smith SGJ, Cox D. Escherichia coli induces platelet aggregation in an FcγRIIa-dependent manner. J Thromb Haemost. avr 2016;14(4):797-806.

25. Ståhl A lie, Svensson M, Mörgelin M, Svanborg C, Tarr PI, Mooney JC, et al. Lipopolysaccharide from enterohemorrhagic Escherichia coli binds to platelets through TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. Blood. 1 juill 2006;108(1):167-76.

26. Vallance TM, Ravishankar D, Albadawi DAI, Layfield H, Sheard J, Vaiyapuri R, et al. Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation. Sci Rep. 3 déc 2019;9(1):18258.

27. Zhang G, Han J, Welch EJ, Ye RD, Voyno-Yasenetskaya TA, Malik AB, et al. Lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway. J Immunol. 15 juin 2009;182(12):7997-8004.

28. Zhao L, Ohtaki Y, Yamaguchi K, Matsushita M, Fujita T, Yokochi T, et al. LPS-induced platelet response and rapid shock in mice: contribution of O-antigen region of LPS and involvement of the lectin pathway of the complement system. Blood. 1 nov 2002;100(9):3233-9.

29. Assinger A, Schrottmaier WC, Salzmann M, Rayes J. Platelets in Sepsis: An Update on Experimental Models and Clinical Data. Front Immunol. 17 juill 2019;10:1687.

30. Matus V, Valenzuela JG, Hidalgo P, Pozo LM, Panes O, Wozniak A, et al. Human platelet interaction with E. coli O111 promotes tissue-factor-dependent procoagulant activity, involving Toll like receptor 4. PLoS One. 2017;12(9):e0185431.

31. Moriarty RD, Cox A, McCall M, Smith SGJ, Cox D. *Escherichia coli* induces platelet aggregation in an FcγRIIa-dependent manner. J Thromb Haemost [Internet]. avr 2016 [cité 8 déc 2021];14(4):797-806. Disponible sur: https://onlinelibrary.wiley.com/doi/10.1111/jth.13226

32. Watson CN, Kerrigan SW, Cox D, Henderson IR, Watson SP, Arman M. Human platelet activation by Escherichia coli: roles for FcγRIIA and integrin αIIbβ3. Platelets. sept 2016;27(6):535-40.

33. Fejes AV, Best MG, van der Heijden WA, Vancura A, Verschueren H, de Mast Q, et al. Impact of Escherichia coli K12 and O18:K1 on human platelets: Differential effects on platelet activation, RNAs and proteins. Sci Rep. 1 nov 2018;8(1):16145.

34. Palankar R, Kohler TP, Krauel K, Wesche J, Hammerschmidt S, Greinacher A. Platelets kill bacteria by bridging innate and adaptive immunity via platelet factor 4 and FcγRIIA. J Thromb Haemost. juin 2018;16(6):1187-97.

35. Tohidnezhad M, Varoga D, Wruck CJ, Podschun R, Sachweh BH, Bornemann J, et al. Platelets display potent antimicrobial activity and release human beta-defensin 2. Platelets. 2012;23(3):217-23.

36. Stahl A l. Lipopolysaccharide from enterohemorrhagic Escherichia coli binds to platelets through TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. Blood [Internet]. 1 juill 2006 [cité 23 août 2022];108(1):167-76. Disponible sur: http://www.bloodjournal.org/cgi/doi/10.1182/blood-2005-08-3219

37. Li C, Li J, Ni H. Crosstalk Between Platelets and Microbial Pathogens. Front Immunol [Internet]. 7 août 2020 [cité 16 déc 2021];11:1962. Disponible sur: https://www.frontiersin.org/article/10.3389/fimmu.2020.01962/full

38. Kraemer BF, Campbell RA, Schwertz H, Cody MJ, Franks Z, Tolley ND, et al. Novel Anti-bacterial Activities of β-defensin 1 in Human Platelets: Suppression of Pathogen Growth and Signaling of Neutrophil Extracellular Trap Formation. DeLeo FR, éditeur. PLoS Pathog [Internet]. 10 nov 2011 [cité 16 mai 2022];7(11):e1002355. Disponible sur: https://dx.plos.org/10.1371/journal.ppat.1002355

39. Tohidnezhad M, Varoga D, Podschun R, Wruck CJ, Seekamp A, Brandenburg LO, et al. Thrombocytes are effectors of the innate immune system releasing human beta defensin-3. Injury [Internet]. juill 2011 [cité 24 août 2022];42(7):682-6. Disponible sur: https://linkinghub.elsevier.com/retrieve/pii/S0020138310008399

40. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature [Internet]. janv 2002 [cité 25 août 2022];415(6870):389-95. Disponible sur: http://www.nature.com/articles/415389a

41. Andersson DI, Hughes D, Kubicek-Sutherland JZ. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. Drug Resist Updat. mai 2016;26:43-57.

42. Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol. sept 2000;8(9):402-10.

43. Kerrigan SW. Platelet Interactions with Bacteria. In: Kerrigan SW, Moran N, éditeurs. The Non-Thrombotic Role of Platelets in Health and Disease [Internet]. InTech; 2015 [cité 18 mai 2022]. Disponible sur: http://www.intechopen.com/books/the-non-thrombotic-role-of-platelets-in-health-and-disease/platelet-interactions-with-bacteria

44. Kerrigan SW, Cox D. Platelet-bacterial interactions. Cell Mol Life Sci. févr 2010;67(4):513-23.

45. Huber M, Kalis C, Keck S, Jiang Z, Georgel P, Du X, et al. R-form LPS, the master key to the activation ofTLR4/MD-2-positive cells. Eur J Immunol. mars 2006;36(3):701-11.

46. Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, et al. CD14 is required for MyD88-independent LPS signaling. Nat Immunol. juin 2005;6(6):565-70.

47. Kappelmayer J, Beke Debreceni I, Vida A, Antal-Szalmás P, Clemetson KJ, Nagy B. Distinct effects of Re- and S-forms of LPS on modulating platelet activation. J Thromb Haemost [Internet]. avr 2013 [cité 20 déc 2021];11(4):775-8. Disponible sur: https://onlinelibrary.wiley.com/doi/10.1111/jth.12151

48. Bessis M. Studies in electron microscopy of blood cells. Blood. déc 1950;5(12):1083-98.

49. White JG, Krumwiede M. Some contributions of electron microscopy to knowledge of human platelets. Thromb Haemost. juill 2007;98(1):69-72.

50. Hannachi N, Lepidi H, Fontanini A, Takakura T, Bou-Khalil J, Gouriet F, et al. A Novel Approach for Detecting Unique Variations among Infectious Bacterial Species in Endocarditic Cardiac Valve Vegetation. Cells [Internet]. 13 août 2020 [cité 27 sept 2022];9(8):1899. Disponible sur: https://www.mdpi.com/2073-4409/9/8/1899

51. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 23 févr 2016;315(8):801-10.

52. Martinod K, Deppermann C. Immunothrombosis and thromboinflammation in host defense and disease. Platelets. 3 avr 2021;32(3):314-24.

53. Greco E, Lupia E, Bosco O, Vizio B, Montrucchio G. Platelets and Multi-Organ Failure in Sepsis. Int J Mol Sci. 20 oct 2017;18(10):E2200.

54. Bonten M, Johnson JR, van den Biggelaar AHJ, Georgalis L, Geurtsen J, de Palacios PI, et al. Epidemiology of Escherichia coli Bacteremia: A Systematic Literature Review. Clin Infect Dis. 8 avr 2021;72(7):1211-9.

55. Pupo E, Lindner B, Brade H, Schromm AB. Intact rough- and smooth-form lipopolysaccharides from Escherichia coli separated by preparative gel electrophoresis exhibit differential biologic activity in human macrophages. FEBS J. févr 2013;280(4):1095-111.

56. Ouyang Y, Wang Y, Liu B, Ma X, Ding R. Effects of antiplatelet therapy on the mortality rate of patients with sepsis: A meta-analysis. J Crit Care. avr 2019;50:162-8.

57. Kiers D, van der Heijden WA, van Ede L, Gerretsen J, de Mast Q, van der Ven AJ, et al. A randomised trial on the effect of anti-platelet therapy on the systemic inflammatory response in human endotoxaemia. Thromb Haemost. 30 août 2017;117(9):1798-807.

58. Keane C, Tilley D, Cunningham A, Smolenski A, Kadioglu A, Cox D, et al. Invasive Streptococcus pneumoniae trigger platelet activation via Toll-like receptor 2. J Thromb Haemost. déc 2010;8(12):2757-65.

59. Kurpiewski GE, Forrester LJ, Campbell BJ, Barrett JT. Platelet aggregation by Streptococcus pyogenes. Infect Immun. févr 1983;39(2):704-8.

60. Berthet J, Damien P, Hamzeh-Cognasse H, Arthaud CA, Eyraud MA, Zéni F, et al. Human platelets can discriminate between various bacterial LPS isoforms via TLR4 signaling and differential cytokine secretion. Clinical Immunology. déc 2012;145(3):189-200.

61. Damien P, Cognasse F, Eyraud MA, Arthaud CA, Pozzetto B, Garraud O, et al. LPS stimulation of purified human platelets is partly dependent on plasma soluble CD14 to secrete their main secreted product, soluble-CD40-Ligand. BMC Immunol. déc 2015;16(1):3.

62. Kojok K, Mohsen M, El Kadiry AEH, Mourad W, Merhi Y. Aspirin Reduces the Potentiating Effect of CD40L on Platelet Aggregation via Inhibition of Myosin Light Chain. JAHA. 4 févr 2020;9(3):e013396.

63. Davì G, Ferroni P. CD40-CD40L interactions in platelet activation. Thromb Haemost. 2005;93(06):1011-2.

64. Judge HM, Buckland RJ, Holgate CE, Storey RF. Glycoprotein IIb/IIIa and P2Y $_{12}$ receptor antagonists yield additive inhibition of platelet aggregation, granule secretion, soluble CD40L release and procoagulant responses. Platelets. nov 2005;16(7):398-407.