RESEARCH ARTICLE

Visualizing the Indefinable: Three-Dimensional Complexity of ‘Infectious Diseases’

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Abstract

Background
The words ‘infection’ and ‘inflammation’ lack specific definitions. Here, such words are not defined. Instead, the ability to visualize host-microbial interactions was explored.

Methods
Leukocyte differential counts and four bacterial species (Staphylococcus aureus, Streptococcus dysgalactiae, Staphylococcus chromogenes, and Escherichia coli) were determined or isolated in a cross-sectional and randomized study conducted with 611 bovine milk samples. Two paradigms were evaluated: (i) the classic one, which measures non-structured (count or percent) data; and (ii) a method that, using complex data structures, detects and differentiates three-dimensional (3D) interactions among lymphocytes (L), macrophages (M), and neutrophils (N).

Results
Classic analyses failed to differentiate bacterial-positive (B+) from –negative (B−) observations: B− and B+ data overlapped, even when statistical significance was achieved. In contrast, the alternative approach showed distinct patterns, such as perpendicular data inflections, which discriminated microbial-negative/mononuclear cell-predominating (MCP) from microbial-positive/phagocyte-predominating (PP) subsets. Two PP subcategories were distinguished, as well as PP/culture-negative (false-negative) and MCP/culture-positive (false-positive) observations. In 3D space, MCP and PP subsets were perpendicular to one another, displaying ≥91% specificity or sensitivity. Findings supported five inferences: (i) disease is not always ruled out by negative bacterial tests; (ii) low total cell counts can coexist with high phagocyte percents; (iii) neither positive bacterial isolation nor high cell counts always coincide with PP profiles; (iv) statistical significance is not synonymous with discrimination; and (v) hidden relationships cannot be detected when simple (non-structured) data...
formats are used and statistical analyses are performed before data subsets are identified, but can be uncovered when complexity is investigated.

**Conclusions**

Pattern recognition-based assessments can detect host-microbial interactions usually unobserved. Such cutoff-free, confidence interval-free, gold standard-free approaches provide interpretable information on complex entities, such as 'infection' and 'inflammation', even without definitions. To investigate disease dynamics, combinations of observational and experimental longitudinal studies, on human and non-human infections, are recommended.

**Introduction**

The word 'infection' has been used for centuries. When the terms 'infection' and 'definition' are searched for, the Web of Science currently retrieves more than 7000 publications. Hence, readers might expect to find a large consensus on the definition of 'infection.' Yet, nothing could be farther from the truth: more than 700 definitions have been proposed for just one disease [1]. Such situation is seen in many diseases [2]. If no agreement can be reached on the definition of a single infectious disease, how can infections be diagnosed?

After 132 years, Koch’s postulates still apply: infectious diseases are caused by microbes, which may be isolated [3]. However, that is only one of many possibilities. Even Koch modified his first postulate: infection differs from colonization [3]. Similarly, a bacterial-negative test is no evidence of an infection-free status: false-negative tests (infections in which no bacterium is detected) may occur due to a variety of reasons [4, 5]. Because infections have at least six expressions [6] and because, historically, diagnosis has been based on a circular fallacy (to select a diagnostic test, the identity of the infecting microbe should be known in advance and, to identify a specific microbe, a specific diagnostic test is needed), it seems that diagnosing ‘infection’ cannot be achieved with classic approaches—a different paradigm is needed.

To visualize infectious diseases, its ‘mirror image’—the immune response—may be considered. While not specific, immunity is not prone to the problems described above [7, 8]. However, both ‘immunity’ and ‘inflammation’ lack a single, agreed-upon definition. Instead of focusing on definitions, here we focus on the data used to diagnose and prognosticate.

While the technology used to collect data is not a major problem, data interpretation is problematic. Interpretation involves several issues, such as: (i) data structuring; (ii) unintended consequences of combinatorial interactions (e.g., errors); and (iii) a topic encapsulated in this question: are host-microbial interactions complex or simple systems?

In clinical medicine, information refers to separate what is different, i.e., to discriminate. Discrimination is the requisite for medical decisions. To decide, data should be transformed into interpretable information [9]. Such process requires data structuring.

To illustrate the difference between data and information, let us consider a 'bio-economical' question: how to sustain life at the lowest cost—that is, how to do more, with less, better and faster? Because the number of pathogens is too large to be known, but the number of immune cell types is very low (up to 5 cell types, according to classic immunology; or less than 300 cell types [10], if we assume that all cell types may participate in immune responses), it follows that a one-to-one relationship (one specialized cell type per each pathogen) is not adequate—if such strategy had been adopted, all creatures would be much larger than the largest elephant,
becoming highly vulnerable to any food shortage. Instead, evolution has conserved small creatures. How can small creatures face so many pathogens with so limited resources?

The answer involves biological combinations [11, 12]. The same element—combined with other element(s)—can perform many (including opposite) functions. For instance, monocytes both promote and destroy neutrophils. Similarly, some cytokines induce and prevent the same immune response [13–16].

While combinations foster survival, they also create problems, such as non-interpretable data and hidden interactions [17]. Unless shown otherwise, biological data may resemble an iceberg: we only see its tip. While valuable information may be hidden, the observed data may be non-interpretable and/or error-prone. For example, when a numerical cut-off is imposed on continuous data (such as cell counts) and discontinuous (discrete) labels are assigned to data points located above or below the cut-off (e.g., infected and non-infected), it has been known, since 1983, that false-negatives and/or false-positives will be generated [18].

Because all elements of the immune system are always interacting and changing, the unit of study is not any one observable data point, but the usually unobserved system [19]. Then, is the immune system simple or complex? Is it internally homogeneous or heterogeneous? Is it composed of only one level or its functions involve multiple levels? The literature indicates that it is complex, heterogeneous, and multi-level [12, 20, 21].

What does ‘complex’ actually mean? First, it means that there are numerous and heterogeneous elements (e.g., cell receptors), which perform different functions [22]. Second, it means that it is composed of many structural levels (e.g., organs). The mathematics that suit one level may differ from that of another level [12]. Such levels also differ in their contents. For instance, human blood only contains approximately 2.2% of all lymphocytes, the bone marrow ~ 10%, the gut between 5 and 20%, and, together, the spleen and lymph nodes contain about 60% lymphocytes [23]. In addition, different relationships may occur: while the CD4/CD8 lymphocyte ratio is about 1.2:1 in lymph nodes, it is 0.3:1 in the spleen [23]. Third, it means that a complex system is interdependent, with communication flows going in all directions [22].

Because the immune system is not a static, internally homogeneous, single-level, single-flow oriented structure composed of independent elements which always have the same meaning (and it may include unobserved interactions), inferences or predictions about the immune system cannot be based on ‘sampling’ any one element. The basic tenets of classic statistical tests (data independence and normality) do not apply to host-microbial interactions [24]. If ‘random sampling’ does not apply to infectious diseases, how can they be assessed?

Uncovering complexity is one alternative. Methods that reveal biological complexity may extract more information than classical approaches [25]. Complex systems are characterized by three properties: (i) emergence, (ii) irreducibility, and (iii) unpredictability [26–28].

Emergence (also known as novelty) refers to the new features revealed only when a complex (system-level) structure is assembled. Irreducibility means that emergence cannot be explained by or reduced to the properties of any one variable. Unpredictability refers to the inability to predict emergence when only ‘simple’ and/or isolated variables are analyzed, e.g., immunoglobulins express emergent properties, which are neither reducible to first principles nor predictable [29]. Similarly, the three-dimensional (3D) functions performed by cell groups (multicellularity) cannot be predicted by 2D models—much less by 1D or tabular formats [30].

To elicit the hidden information complex systems tend to have, dimensionless indicators can be considered [31, 32]. Such indicators, together with data structures designed to possess some desirable properties, could provide pattern recognition-based information [33, 34].

Here we explored a proof-of-concept derived from the previous considerations, which was evaluated with microbiological and leukocyte data collected from animals. Two questions were
asked: (i) do host-microbial interactions reveal complex properties?, and (ii) if demonstrated, can such properties provide information that facilitate medical decisions?

Materials and Methods

Animals

A cross-sectional and randomized study was performed with 611 bovine milk samples collected from the same number of mammary glands in mid-lactating Israeli Holstein cows. Samples were used to identify and quantify leukocytes, and conduct microbial cultures (S1 Dataset). No bacteria were found in 464 of such samples, and three bacterial species were isolated from the remaining 147 quarters: (i) \textit{Staphylococcus aureus} (\(n = 31\)); (ii) \textit{Staphylococcus chromogenes}, (\(n = 74\)); and (iii) \textit{Streptococcus dysgalactiae} (\(n = 42\)). Seventy eight culture-negative tests proceeded from mammary glands that, one month earlier, yielded \textit{Escherichia coli} isolates. \textit{E. coli}+ cows were treated, for 3 days, with antibiotics (ABIC Biological Laboratories, Teva Ltd., Israel) and anti-inflamatory drugs (Norbrook Laboratories, Ireland). No bacterium was isolated in the previous month from milk of the remaining cows. Data analyses were conducted twice: not considering and (only in the case of animals previously \textit{E. coli}-positive, which were treated) considering prior information. While cross-sectional in a strict sense, the design used provided more information than classic cross-sectional designs because it also considered prior data.

This study was approved by the Institutional Animal Care Committee of the Israeli Agricultural Research Organization of the Volcani Center and the Institutional Animal Care and Use Committee of the University of New Mexico, United States (protocol 13-101022-T-HSC).

Bacteriological and leukocytes count methods

Culture and molecular procedures with a detection limit estimated a 10 colony-forming units/ml were performed as described before [35]. Negative samples were re-tested with a kit that extracts bacterial DNA using universal 16S primers 4F and 801R (Milk Bacterial DNA Isolation Kit, Norgen Biotek Corp., Ontario, Canada [36]). Total milk cell counts/ml (‘somatic cells’ counts or SCC) were determined with a Fossomatic 360 counter (Foss Electric, Hillerod, Denmark). Using the technique and monoclonal antibodies anti-bovine CD18, CD4, CD8, CD14 and G1 described earlier [37], each leukocyte was identified and its percentage calculated using a FACSCalibur cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA). Because SCC include epithelial cells, leukocyte counts were determined as CD18\(^+\) counts/ml.

Leukocyte data structuring

Primary leukocyte data on lymphocytes (L), macrophages/monocytes (M), and neutrophils (N) were used to detect data subsets that, in 3D space, displayed distinct patterns (e.g., perpendicular data inflections). To that end, a three-step method was implemented: (i) dimensionless indicators were created, which expanded the number of data structures available for analysis; (ii) 3D data structures lacking distinct patterns were deleted; and (iii) using leukocyte and bacteriological data, the biological validity of each distinct data subset was explored. This process was facilitated by a proprietary algorithm that creates dimensionless indicators (combinations of two or more cell types as ratios or products derived from counts or percentages, alone or combined). Biological validity was supported when two or more data combinations identified two or more subsets, which displayed non-overlapping data distributions and differed in discrete classifiers (e.g., when culture-negative observations predominated in one data subset and culture-positive data points predominated in the other subset).
Data analyses

Dimensionless indicators (DI) were generated as described elsewhere [31, 32]. DI were temporary guides used to detect distinct data subsets. Because DI can include many, internally complex relationships (e.g., the \( ([\text{M\%} \times \text{N\%}] \times [\text{M counts} \times \text{N counts}]) / ([\text{MC/N}] / [\text{N\%+M\%}] / \text{L\%}) \)), and the patterns they generate are not the result of any one indicator but the overall 3D interaction, such patterns cannot be predicted. Consequently, the internal structure of individual DIs — identified here with letters expressed in italics, e.g., \( \text{BAL} \) — was not described. Instead, the data contents of subsets detected by DI were described. When one or more data inflections distinguished two or more data subsets, the L, M, and N counts or percentages of each subset were explicitly analyzed. Such evaluation also explored indices that reflect either early anti-microbial responses (e.g., the neutrophil/lymphocyte \( [\text{N/L}] \) ratio, which increases in early responses) or later responses (e.g., the mononuclear cell/\( \text{N} \) \( [\text{MC/N}] \) ratio, which increases in the resolution phase) [38, 39]. Statistical analyses of medians (Mann-Whitney test) or proportions (Chi square test), as well as 3D plots, were conducted or created with a commercial package (Minitab Inc, State College, PA, USA).

Results

The total leukocyte count revealed a threshold around (log) CD18+ count = 5. While culture-negative results predominated below that value, 78 culture-negative observations were found above such threshold (Fig 1A). Prior information seemed relevant: had prior information been considered (animals previously \( \text{E. coli} \)-positive, which were culture-negative when leukocytes were measured), negative observations observed at (log) CD18+ counts > 5 would become positive (Fig 1B). Prior information demonstrated that CD18+ counts were better indicators than the SCC: while a non-trivial number of culture-negative and —positive observations overlapped when SCC were measured (red box, Fig 1C), CD18 counts showed negligible data overlapping (Fig 1C).

The fact that prior information is not always available prompted additional questions, such as: (i) is it possible to distinguish immune subcategories, if they exist?, (ii) can false culture-negatives and false culture-positives be detected?, (iii) can the same microbe induce different immune responses?, and (iv) is statistical significance synonymous with discrimination? None of such questions were answered by molecular data: the CD4+ lymphocyte percent, the CD8+ L%, and the CD4/CD8 ratio failed to distinguish culture-negatives from-positives, even when statistical significance was achieved \( (P<0.03, \text{Fig 2A}) \). Similarly, cellular data (L, N, or M percents) did not discriminate, even when median values differed statistically between the culture-negative and -positive groups (blue circles, \( P<0.03, \text{Fig 2B} \)). Regardless of prior information, neither cellular percentages nor indices that captured interactions involving two or more cell types (supra-cellular relationships) differentiated bacterium-specific infections: overlapping data distributions were observed (Fig 2C and 2D). Thus, regardless of the scale investigated, neither leukocyte- nor bacterial-related classes were distinguished when immunity was assumed to be a simple system, i.e., when L, M, or N data points were assessed separately.

The use of counts, even when analyzed in 3D space, did not detect hidden interactions. For instance, when CD18+ counts were measured together with the N/L and MC/N ratios (indicators that characterize the early and late anti-microbial immune phases, respectively), both high N/L and high MC/N observations were associated with culture-negative and -positive observations (brown and blue boxes, respectively, Fig 3A). Discrimination was even poorer when current information was considered (Fig 3B).

In contrast, when dimensionless indicators were used and prior information was considered, emergence was demonstrated: four new data subsets were detected (Fig 4A and 4B). Data
Fig 1. Discriminant diagnostic ability of cell counts. Cell counts collected from 611 bovine milk samples are reported. When CD18+ and neutrophil (N) counts were assessed and cultures were conducted, culture-negative observations predominated below (log) 5 CD18+ counts/ml; however, numerous culture-negative data points were also observed above such threshold (A). Culture-negative observations above (log) 5 (CD18+ 1000 counts/ml) corresponded to animals previously *E. coli*-positive. If prior information were
inflections characterized such subsets as: (i) culture-negative, (ii) predominantly culture-negative (including a few culture-positive data points), (iii) culture-positive, and (iv) predominantly culture-positive (including a few culture-negative observations, arrows, Fig 4C).

When only current information was considered (i.e., when previously *E. coli*-positive cows were regarded as culture-negative—their status at testing time), data combinations improved discrimination. Metrics that, before, did not differentiate (Fig 2C and 2D), when re-grouped distinguished (with minor data overlapping) between culture-negative/mononuclear cell-predominating (MCP) subsets and culture-positive/phagocyte-predominating subsets (PP, boxes, Fig 5A). Additional indices (which included ratios and products) discriminated two phagocyte-related subsets: one totally and one only partially associated with positive cultures (red and blue symbols, Fig 5B).

Additional emerging patterns detected observations suspected to be false. When bacterial and leukocyte data were considered, one false-positive (FP or culture-positive/MCP) observation and five false-negatives (culture-negative/PP observations) were found (Fig 6A and 6B). False-negatives were located within the range of culture-positive observations, but far from the location of culture-negative/MCP data points (Fig 6C). The FP observation was located within the data range of culture-positives, but far from the location of culture-positives (Fig 6C). When prior information was not considered, additional FNs were detected (box, Fig 6D).

When prior information was considered, immunologic profiles supported the FN/FP classification: FNs showed the lowest L% (and many FNs displayed lymphocyte values below the lower limit of culture-negative data points), while the FP observation revealed one of the highest L% values among all data points (Fig 7A). FN and FP were also detected when cows previously infected with *E. coli* were assessed according to their status at the time of testing, that is, as culture-negative animals (Fig 7B).

Emergence was also documented when only culture-positive data points were analyzed: three microbial subsets were then detected (Fig 8A). Such subsets were distinguished by monocyte/lymphocyte (M/L) ratio values and/or L percent values (Fig 8B).

When dimensionless indicators were used, both *E. coli* and *S. chromogenes*-positive observations revealed a data inflection. Such inflection differentiated two data subsets (Fig 9A and 9B).

While most *S. chromogenes*-positive observations (40/74 or 54%) were found within the horizontal subset (mainly characterized by high lymphocyte percents and negative cultures), most *E. coli*-positive data points (61/78 or 78%) were within the vertical subset (characterized by positive cultures and high neutrophil percents, Fig 10A and 10B). Because the same bacteriological tests were used twice, the high N percents associated with negative bacterial cultures (observed 61 times in the later assessment) were not likely to be false (*E. coli*-negative) tests. The proportion of *S. chromogenes*-positive observations within the horizontal subset was two-fold higher than that of *E. coli*-positive observations (45.9 vs. 21.8%, respectively, *P*<0.002, Chis square test). Findings showed two contrasting pictures: leukocyte profiles were similar...
Fig 2. Discriminant diagnostic ability of simple or low-complexity indicators. Neither molecular (cell-surface marker-related) nor cellular indicators (expressed as leukocyte percentages) distinguished culture-negative from-positive observations (A, B). While median values reached statistical significance (blue circles, \( P < 0.03 \), Mann-Whitney test, A, B), overlapping leukocyte data distributions were observed between the culture-negative and -positive groups (blue boxes, A, B). Such lack of discrimination remained when low-
complexity indicators (ratios that measured interactions involving two or more cell types) were considered, regardless of current information on bacterial status (C) or prior information (i.e., assuming as culture-positive all previously E. coli-positive animals, D).

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within subsets (regardless of whether a microbe was isolated or not) but dissimilar across subsets—even when the same microbe was involved. For instance, the median L % for the vertical subset was 4.3 and 5.1 (for E. coli and S. chromogenes, respectively) and, for the horizontal subset, it was 27.5 and 22.9% (for E. coli and S. chromogenes, respectively). Neutrophil and phagocyte (M and N) median percents revealed similar patterns (Fig 10A and 10B). Yet, the L, N, and phagocyte medians differed across subsets ($P<0.001$, Mann-Whitney test). Culture-negative data points displayed patterns similar to those of S. chromogenes- and E. coli-positive groups (Fig 10C). While statistical significance was achieved when subsets were compared, it did not discriminate: a non-trivial number of observations overlapped across subsets (blue boxes, Fig 10A–10C).

The sensitivity and specificity of data subsets identified by dimensionless indicators were estimated (Fig 11A and 11B). While the apparent specificity of a culture-negative subset was 91.4% (96/105), because such subset included one FP, its actual value was 92.4% (97/105, Fig 11A). In a subset perpendicular to the previous one, 37 observations were found, of which 34 yielded bacterial cultures (an apparent sensitivity equal to 34/37, or 91.9%). However, because 3 FNs were included, its actual value was 100% (37/37, Fig 11B). Such inferences were biologically validated: higher L% and lower N% were displayed by the specificity subset than by the subset used to estimate sensitivity (Fig 11C). Because this study lacked temporally explicit data, dynamics could not be measured. Because sensitivity and specificity do not account for time and an observational study, such as this, may include several time-related immune responses, the sensitivity and specificity associated with almost ¾ of all data points remained undetermined.

A double interaction (the $[L/M] / [N/L]$ ratio, Fig 12A) showed a third data subset (here identified as ‘other’) in addition to the ‘horizontal’ and ‘vertical’ subsets observed in Fig 11A and 11B.

Fig 3. Discriminant diagnostic ability of simple or low-complexity indicators measured in 3D space. Cell count-based analyses, even when 3D patterns were considered, did not discriminate: CD18+ counts, together with the N/L and MC/N ratios, failed to distinguish immuno-microbial subsets when prior (A) or current (B) information was considered.

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While partially sharing [L/M] / [N/L] ratio values with the ‘vertical’ subset, the ‘other’ subset differed from the ‘horizontal’ subset and—at least when the L/N ratio was considered—also from the ‘vertical’ subset (Fig 12A). Therefore, at least two PP subsets were distinguished. However, when plotted in 3D space, the leukocyte variables did not show distinct limits: the ‘other’ subset partially overlapped with the remaining ones (Fig 12B). That limitation could have been prevented had the data been structured as a single (one data point-wide) line of observations (as
shown in Fig 12C) and temporal data had been available. When two or more longitudinal data points are measured within a single line of observations, an arrow that connects any pair of points will provide temporal data directionality, indicating whether the most recent observation is moving toward the disease-negative or -positive pole of the data structure.

Discussion

This study investigated a novel method that explores host-microbial interactions. To that end, bovine immune responses against intra-mammary infections (IMI) were explored. IMI cause milk production loss and changes in its composition [40–43]. IMI also epitomize diagnostic problems observed across species—humans included. For example, while PCR tests used to detect pathogens in bovine milk seem to possess 100% sensitivity and specificity [44], in sub-clinical cases, the sensitivity of PCR tests decreases substantially [45]. The false-negative rate of milk
cultures ranges from 9 to 37% [46]. PCR-related false-positive test results can be due to numerous reasons, such as non-viable bacteria and contamination with extra-mammary bacteria [45]. Errors can also occur when bacterial clearance coexists with protracted inflammation [47].

Fig 6. Detection and evaluation of observations suspected to be false (emergence II) Emergence was also expressed as observations suspected to be false. Such detection was elicited by dimensionless indicators (hypothetical indices derived from products or ratios of leukocyte data, which assess numerous relationships, e.g., BAS, AM, BAP). Dimensionless indicators detected two subsets of culture-negative data (A). Because culture-negative data subsets were separated by the culture-positive cluster, one subset was suspected to be false-negative (FN culture results, B). A similar contrast led to suspect that one culture-positive observation was a false-positive (FP): it was located within the culture-negative cluster but far from the location of culture-positives (B). Such inferences did not depend on the set of dimensionless indicators analyzed or whether current or prior information was considered: when a separate set of indicators was analyzed, it showed similar patterns (C) and, when only current information was considered, patterns remained, differing only in the number of FNs observed, which increased (red polygon, D).

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New or emergent patterns were detected when complex data structures were generated and data subsets were differentiated (Figs 4–12). Because non-structured data did not display such patterns (Figs 1–3), emergence was neither reduced to nor explained by the properties of any one variable; that is, both irreducibility and unpredictability, in addition to emergence, were demonstrated [26–28]. Emergent patterns are not truly new: they are so called because such patterns are rarely observed. Such patterns supported two major messages: (i) hidden interactions can be uncovered, provided that the complexity of host-microbial interactions is explicitly explored; and (ii) alone, neither bacteriological tests nor leukocyte profiles are diagnostic.

To detect hidden interactions, a proof-of-concept was developed, which pursued two goals: (i) to increase the number of data shapes to be investigated (so the chances of capturing hidden patterns were increased), and (ii) to prevent an excessively large number of combinations (so the procedure could be conducted in real time). To achieve the first aim, hypothetical
\( \text{(dimensionless)} \) indicators were created and used to uncover data subsets. To validate such subsets, biologically interpretable data were analyzed. Such approach elicited data points suspected to be false and differentiated immuno-microbial classes, identifying five limitations of bacteriological tests and leukocyte profiles:

- **i.** disease is not ruled out by negative bacterial tests (e.g., even after antibiotic and anti-inflammatory treatments, culture-negative data of animals previously \( E. \ coli \)-positive were associated with phagocyte-predominant immune responses);
- **ii.** low total cell counts can coexist with phagocyte-rich immune responses;
- **iii.** neither positive bacterial isolation nor high cell counts demonstrate recent disease (e.g., one \( S. \ chromogenes \)-positive data subset showed high lymphocyte percents, which displayed values similar to those of culture-negative observations);
iv. **statistical significance is not synonymous with discrimination** (this study demonstrated, seven times, that comparisons that achieved statistical significance failed to show non-overlapping data distributions); and

v. **hidden relationships cannot be detected when simple (non-structured) data formats are used and statistical analyses are performed before data subsets are identified.**

Because two phagocyte-rich data subsets were observed, the classic dichotomy of infection-related classes (the presence of only two [one bacterial-positive and one bacterial-negative] classes) was rejected [48]. Because the observed data subsets were neither cell type- nor bacterium-specific, 'simple' models were also rejected [49].

Yet, the analysis of complexity was not complicated: interpretation was achieved without prior knowledge [26, 50]. Because inferences were based on redundancy (Figs 9, 11), interpretations did not depend on any ‘gold standard.’ That is a methodological advantage because, unlike physics (where units of measure can be standardized), in biology, definitions of ‘infection’ and ‘inflammation’ (as well as those on ‘standards’) have not led, so far, to unambiguous and constant interpretations. Because any one biological element (e.g., a cytokine, a cell, a cellular biomarker), over time and in conjunction with other elements may have or perform more than one meaning or function, this pattern recognition-based approach may provide a bio-temporal context to evaluate biomarkers and pharmaceutical drugs. For instance, longitudinal-experimental studies conducted with data structures similar to those here explored could determine the net
effect of a drug or the function of a biomarker. By focusing on the complexity and dynamics of immuno-microbial relationships, such studies could elucidate when (in which disease stage) a biomarker is expressed or a drug induces an observable effect. Because complex biological systems (such as diseases) are well-conserved, the temporal ('early' or 'late') sequence of biological

**Fig 10. Evaluation of immuno-microbial subsets.** When the subsets reported in Fig 9 were explored with leukocyte data, the horizontal subset revealed significantly higher L%*, regardless of whether S. chromogenes-positive only, (previously) E. coli-positive only, or culture-negative only data were assessed (A-C). Similarly, the vertical subset displayed significantly higher N % and phagocyte (P or N+M) percentages than the horizontal subset (P<0.001, Mann-Whitney test). Yet, statistical significance did not result in discrimination; overlapping data distributions were found between subsets, in all assessments (A-C).

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interactions can be assessed regardless of chronological units (hours/days) and not considering whether the tested individual is a slow or fast responder [33]. Because such systems display graphic (three-dimensional) cut-offs, interpretations do not depend on numerical cut-offs—which can induce false-negative and -positive results [18].

Because the labels 'early' and 'late' immune responses used here were only tentative, longitudinal studies are needed to confirm such temporal classifiers. Such new studies (conducted in non-human and human species) could also explore whether cellular and humoral factors, if simultaneously investigated, increase discrimination.

At least four needs justify future studies on complex host-microbial interactions: (i) earlier diagnosis, (ii) enhanced sensitivity, (iii) diminished error rates, and (iv) new information. If computerized, appraisals of such relationships could be more rapidly implemented than

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**Fig 11. Detection and evaluation of new inflammatory responses (emergence V).** Robustness and redundancy were documented; patterns similar to those reported in Fig 9 were obtained when different indicators were utilized, which detected one horizontal subset (mainly composed of culture-negative observations) and one vertical subset (mainly composed of culture-positive observations, A, B). The median L, N, and phagocyte percents of such subsets differed, reaching statistical significance (blue circles, P<0.001, Mann-Whiney test, C).

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bacteriological tests. Complex data structures are more sensitive than classic metrics, e.g., ratios grow larger or faster than percents [34]. When a single line of observations is created, data can change only within a line, facilitating interpretations that do not require confidence intervals. When distinct data patterns are observed, bacterial isolates that induce different immune responses (such as *S. chromogenes* isolates) can be distinguished without additional tests.
Because anti-microbial immunity involves, at least, four levels of complexity [51], it is suggested that neither ‘infection’ nor ‘inflammation’ is definable. However, if measured together, both concepts can be described: based on graphic patterns, each data point can be interpreted according to the subset it belongs to. To uncover complex host-bacterial interactions, any data combination can, probably, be used, provided that: (i) the data structure reveals desirable properties (e.g., a single line of observations, distinct patterns, temporal data directionality); (ii) subsets are distinguished and validated; and (iii) statistical tests are conducted after (not before) data subsets are detected. Such propositions may apply to human infections.

Conclusions
The visualization of leukocyte-bacterial interactions can improve our understanding on infectious diseases. A proof-of-concept that assessed the complexity of such relationships uncovered patterns usually unobserved, which revealed possible solutions to several problems. To explore the dynamics of infectious diseases, additional studies, of longitudinal nature, are needed.

Supporting Information
S1 Dataset. Data analyzed in this study. (XLS)

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Author Contributions
Conceived and designed the experiments: GL SEB ALR. Performed the experiments: GL SEB ALR. Analyzed the data: GL SEB ALR. Contributed reagents/materials/analysis tools: GL SEB ALR. Wrote the paper: GL SEB ALR.

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