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Review

Polymicrobial consortia in the pathogenesis of biofilm vaginosis visualized by FISH. Historic review outlining the basic principles of the polymicrobial infection theory

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ABSTRACT

The manuscript disputes the exclusive mono-infectious way of thinking, which presumes that for every infection only one pathogen is responsible and sufficient, when infectious vectors, close contact and reduced immunity meet. In situations involving heavily colonized anatomical sites such an approach often ends in insoluble contradictions. Upon critical reflection and evaluation of 20 years research on spatial organization of vaginal microbiota it is apparent, that in some situations, pathogens may act and operate in permanent, structurally organized consortia, whereas its individual components may be innocuous and innocent, failing to express any pathogenic effect. In these cases, consortia are the true pathogens responsible for many infectious conditions, which usually remain unrecognized as long as improperly diagnosed.

The structure of such consortia can be unraveled using ribosomal fluorescence in situ hybridization (FISH). FISH methodology, that not only offers an ex vivo opportunity to recognize bacterial species, but provides unique physical insight into their specific role in the pathogenesis of polymicrobial infections. Ribosomal FISH technique applied to both, women with bacterial vaginosis (BV) and their male partners, has added significantly to our understanding of the pathogenesis of this condition and contributed to appreciating the mechanisms of polymicrobial, community-based infection, potentially leading to therapeutic advances.

1. Introduction

Contemporary infection science reflects viral, bacterial, protozoal, fungal and parasitic diseases, all are primarily determined as mono-microbial infections. Polymicrobial infections in which each component acts as an indispensable part of a microbiota-team, are presently neither known nor even widely recognized as a real possibility.

In the early 1930s, Rosebury proposed that for some infections a close cooperation of microorganisms is necessary. Investigating cases of contagious gingivitis he believed he had isolated joint participants, four of which were necessary for transfection and he called them a “pathogenic quartet” [1]. Unfortunately, this hypothesis, could not be validated by other researchers, who used diverse isolates of pure cultures applied in arbitrary mixtures. Having failed to identify these experimentally, his ideas found no support and were largely forgotten. However, modern molecular genetic-based techniques such as 16/23S targeted ribosomal fluorescence in situ hybridization methods (FISH), which specifically visualize individual bacterial species in although fixated, still natural *in vivo* mixtures, clearly demonstrated that bacteria infecting mucosal surfaces in diseases such as tonsillitis [2], appendicitis [3], colitis [4], and vaginal disorders [5], are not randomly combined, but are rather often assembled in stable, structurally organized consortia, each component of which is indispensable for its resultant pathogenic outcomes. We postulate that such consortia cannot be transferred as individual participants or mechanical mixes of their pure cultures, and need to be relocated collectively in a way that maintains their natural composition and structure and should nevertheless be accepted as actual “individual infectious agents”. This review presents the case of bacterial vaginosis (BV) as the ultimate example of a polymicrobial infection. The manuscript summarizes the available data on polymicrobial involvement in BV, discussing contemporary concepts and emerging perspectives.

2. Databases and FISH protocols used for review

The peer reviewed data dealing with the history, clinical and microbiome studies in patients with BV were based on publications listed in PubMed, WOS, Scopus, Embase and the clinical experience of the authors. The survey on species-specific arrangement of individual microbial groups in vaginal discharge summarizes data revealed by a methodology utilizing fluorescently labeled, 16/23S rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization (FISH) in the Laboratory for Polymicrobial infections at the Charité University Hospital, Berlin Germany.

Vaginal biopsies and smears from women with BV and related symptoms treated in Friedrichshain, Vivantes, Charité (all in Berlin) and Wuppertal hospitals have been regularly investigated at the Charité Hospital since 2004.

FISH microscopy is not suitable for large scale surveys and can be performed only in selected patient groups. Despite the significant number of patients and samples evaluated thorough the years (2000 and 8000, respectively), most of the data were evaluated separately and published in several small sample sized studies, spread over the years. Each of these reports was dedicated to specific aspects of the disease and unsuitable for full overview. Furthermore, the linguistic reports of microscopic findings are primarily descriptive and the terminology used was not initially standardized. The fluorescence images are colorful and extremely complex such that in early studies it was impossible to establish a difference between what was just impressive and what was significant. While reproducing as accurately as possible what was visible, the same processes continued to be described using alternative terminology in subsequent publications, thus making the comparison of results difficult to follow. Nevertheless, an optimal terminology was gradually developed and since 2019, several 16/23S ribosomal FISH laboratories for microbial diagnostics of vaginal disorders and using the same Berlin methodology have been opened consecutively in Saint-Petersburg and Moscow, considerably increasing the comparable database. This has allowed us to unify, classify and peer review the FISH findings in vaginal disorders raised and reported in the last 20 years.

3. Discovery and pitfalls in bacterial vaginosis

In 1955, Gardner and Dukes described vaginal epithelial cells densely covered with short bacterial rods in women with increased vaginal discharge, unpleasant fishy odor, and other vulvovaginal symptoms. These cells could be seen in wet mount microscopy and even better on Gram stain. Since such cells were absent in healthy women and the transfer of vaginal discharge from symptomatic women caused similar symptoms, the authors regarded them as a diagnostic clue for the newly discovered vaginal infection, naming

them “clue cells” [6]. Since then, clue cells have become one of the most important and undisputable microscopic criteria for the disease, which was eventually called “bacterial vaginosis”, highlighting the lack of visible inflammation and enormous numbers of bacteria on microscopy [6–9]. In addition, bacteria most frequently isolated and found in high concentrations in vaginal discharge in women with BV were called *Gardnerella vaginalis* sensu lato. The designation of BV as an infection was less straightforward and more complex, as many facts seemed to contradict this assumption: though in low concentrations, *G. vaginalis* could be isolated from up to 50% of healthy women [7]. Also, no isolated strains of *G. vaginalis* were able to definitively initiate disease upon transfection. Pathogens other than *G. vaginalis* were not identified. We now recognize that the microbial diversity in BV is extremely high, especially when measured with modern, culture-independent molecular-based methods. However, except for *Gardnerella* spp. none of the other potential pathogens could be found in all cases of BV [5,10].

Following the spirit of the times Gardner and Dukes declared that the microorganism isolated (then called *Haemophilus vaginalis*) was the monoetiologic agent responsible for non-specific vaginosis (now known as BV). To demonstrate pathogenicity, they considered whether the organism satisfied Koch's postulates [6]. Koch's postulates were developed to identify the monoetiological agents of diseases such as anthrax and tuberculosis. However, the postulates have their limitations, and are not currently relevant because they do not apply to viral disease, asymptomatic carrier status or polymicrobial consortia. The 3rd postulate states, “the bacterium, in pure culture, must, when inoculated into a susceptible animal give rise to the disease.” Gardner and Dukes inoculated 13 volunteers (who were free of the disease) with pure cultures of *H. vaginalis* one subsequently developed clinical signs and symptoms. The organism, recovered in pure culture, had completely replaced the existing vaginal flora. Two other women had the organism recovered in culture but had no signs or symptoms of disease. Ten women failed to develop clinical evidence of disease or positive cultures. Nevertheless, Gardner and Dukes felt this was sufficient to satisfy Koch's 3rd postulate. In a subsequent attempt to confirm pathogenicity, 15 volunteers (screen negative for other genital tract infections), were inoculated vaginally with the vaginal secretions of donors. Eleven of these developed disease which supported the later view of others, that since vaginal secretions from donors are more successful in causing disease rather than pure cultures [6], *G. vaginalis* as it was later to become known, probably acts synergistically with other micro-organisms to cause BV [11,12]. As the lyrics of the song, popularized by the singer Pearl Bailey in 1952, “it takes two to tango”.

Moreover, antimicrobials are not always curative and often do not avoid recurrence of BV [13–15]. Although the bacterial load of *Gardnerella* spp. and other ‘bacterial vaginosis associated bacteria’ (BVAB) is 1000-fold increased [16,17], antimicrobials frequently help only on a temporarily basis, and the condition, both symptomatic or asymptomatic, relapses in most cases soon after treatment [18,19]. Furthermore, whereas microscopy of vaginal smears from healthy women reveals the presence of large Gram-positive lactobacilli, no such rods are visible within the dense Gram variable bacterial masses typical for BV [20]. BV is therefore interpreted as dysbiosis induced by disappearance of protective lactobacilli and replacement by *Gardnerella* spp. and pathogenic BVAB [21]. However, no specific reason for such dysbiosis was ever found and the results of BV treatment with *Lactobacillus* spp. supplements including locally administered yoghurt, buttermilk or lactic acid and other probiotic preparations were inconsistent [22–24].

When the loss of protective lactobacilli is a decisive part of the problem, one could assume, that sex with different healthy partners could be advantageous. It can be theorized that at least some sexual contacts with healthy partners would lead to recolonization with the protective or “good” lactobacilli, which were allegedly lost. However, epidemiologic data strongly contradict this assumption. BV is the most common genital disease in women of sexually active age [25] and its incidence is highest in women at high risk for sexually transmitted infections including commercial sex workers [26,27].

An interpretation of BV being the result of sperm degradation, which changes the vaginal milieu promoting *Gardnerella* spp. overgrowth [28], cannot explain the disease persistence over years and its frequent recurrence even in the absence of sexual activity, nor the high BV prevalence rates in women practicing sex with women. In this risk group, the association between BV and first sexual contact, change of sexual partner (especially if frequent), and contact with a BV-positive partner is well documented [29]. Males can be involved in BV transmission, but, as far as we are aware, there have not been any consequences identified for male health due to BV.

Sequencing studies confirm that asymptomatic male partners of BV patients have an abundance of BVAB in the subpreputial space and distal urethra, which serve as reservoir for pathogens and hence infection or reinfection [30]. Besides the lack of predominant lactobacilli, a massive influx of pollutants may cause dysbiosis. Considering the anatomic proximity between the anus and vagina, poor genital hygiene is often blamed, yet no individual hygiene measures have been shown capable of reducing the recurrence of BV [31].

The ambiguity of BV microbial pathogenesis is not trivial. Moreover, without understanding the disease nature, it is impossible to define what we are looking for to establish diagnosis and what we are actually treating. Arbitrary interpretations and poor comparability of results are therefore inevitable.

Most often Amsel criteria, Nugent and Hay/Ison score, multiplex polymerase chain reaction (PCR) and next generation sequencing (NGS) are used to diagnose BV [32,33]. The Amsel criteria are still regarded as the gold standard in clinical practice, despite growing dispute, and support evaluation of suspicious symptoms by gynecological examination plus microscopy. They point to BV if at least three out of following features are present: 1) homogeneous, grayish-white vaginal discharge, 2) pH value > 4.5, 3) a fishy amine odor (ready or upon addition of 10% potassium hydroxide), 4) and detection of clue cells by wet mount microscopy [34]. Nugent score (and other similar techniques) assess bacterial morphotypes using Gram stain microscopy according to a formally predetermined protocol. PCR and NGS methods characterize the diversity of the vaginal microbiota [17,35].

While the Amsel criteria are most useful in the clinical evaluation, they require on site microscopy, availability of minimal laboratory equipment with chemical reagents and, most importantly laboratory skills and experience of the gynecologist. Accordingly, the vaginal samples are often sent to external laboratories, where clue cells are enumerated by Gram stain in combination with other methods. Outsourced microscopy, multiplex PCR and NGS ease the task of the clinician without increasing the diagnostic relevance of direct clinical investigation backed by wet mount microscopy [36–38]. The capabilities of modern microbiologic techniques are enormous with respect to microbial identification. More than 500 species can be identified within individual vaginal microbiomes [39]. However, since no disease specific pathogens are yet established, and occurrence of BVAB organisms in health and disease overlaps broadly, a straightforward diagnosis is not possible [17,35,37]. The diversity of detected bacteria has to be relegated to purely descriptive criteria, being thus unable to bring additional information exceeding the statement or justification of the ‘gold standard’.

4. Spatially structured consortia and the polymicrobial nature of BV

Ribosomal RNA gene-based fluorescence in situ hybridization has enhanced our perception of BV substantially [40]. The historical assumption that the vaginal epithelium is colonized by adherent lactobacilli could not be confirmed. In biopsy material obtained from healthy women, the vaginal epithelium was free of bacteria. Microorganisms were seen exclusively in the slime above the epithelial surface (Fig. 1A,a) and were non adherent. Conversely in BV, bacteria were mainly concentrated in a biofilm tightly attached to the vaginal epithelium. The concentration of bacteria in slime was lower than within adherent biofilm [5]. Bacteria were densely packed within the biofilm reaching concentrations of 10^{10}/mL. Bacterial concentrations in adjacent slime outside of the biofilm dropped to less than 10^{6}/mL even in regions located between single, free-laying clue cells as can be seen in Fig. 1c.

The adherent biofilm was primarily comprised of densely packed *Gardnerella* spp. cohesively stuck to each other, and offering a habitat for a large variety of other species often including lactobacilli, *Fannyhessea* (*Atopobium*) *vaginae* and others. In many cases of BV with a demonstrable *Gardnerella* spp. consolidated biofilm, lactobacilli were observed adherent to the vaginal surface (Fig. 1B,b). Such intense adherence of lactobacilli or any other microbial groups was never detected in healthy women [41].

Fig. 1C,c shows a Gram stain of a vaginal biopsy from women with BV, and the corresponding FISH microphotograph of hybridization with the Cy5-Gard probe (*Gardnerella* spp., dark red fluorescence). It can clearly be seen that the clue cells do not arise *de novo* but rather when shedded from the epithelial surface are already completely covered by a previously fully established biofilm.

In material obtained from vaginal swabs, only vaginal discharge with suspended desquamated epithelial cells and bacteria are available for investigation, while the on-site relationships of bacteria to vaginal epithelium is invisible. Other limitations include the fact that the concentrations of bacteria and the disintegration of epithelial cells in samples progresses unpredictably during the transport to the laboratory and the uneven and heterogeneous distribution of material on glass slides when the sample is prepared for microscopy. Within single smears, the transient findings between low and high bacterial accumulation and between unaffected epithelial cells and heavily affected cells may be more numerous than the 'unmistakable' extremes. The morphologic appearance of polymicrobial mixes utilizing light microscopy is often misleading. It is impossible to differentiate *Lactobacillus iners* and *Gardnerella* spp. with any of the available stains [42]. The same applies to many other bacterial groups, especially if they are densely packed to confluent, mostly Gram-labile appearing muds. Interpretation biases are inevitable, even when it comes to such striking and seemingly simple phenomena as clue cells. It is therefore not surprising that despite broad acceptance as the most critical diagnostic criterium and more than 60 years since its first description, no consensus exists even about what should be regarded as a clue cell [43]. A precise definition and consistent precept to measure clue cells is lacking. Confronted with deviant microscopic pictures, each investigator may classify the findings in different ways.

Using ribosomal FISH, we reexamined vaginal swab samples from 500 women. They were collected from 42 gynecologic practices and sent to routine laboratories. In all these women, BV was diagnosed clinically (Amsel criteria) and clue cells were confirmed by light microscopy. Spatial distribution was assessed for *Bifidobacteriaceae*, *Gardnerella* spp, *Fannyhessea vaginae* (*Atopobium*); low G + C (guanine + cytosine) bacteria, lactobacilli, *L. iners*; *Lactobacillus crispatus*, *Gamma-Proteobacteria*; and *Enterobacteriaceae*, *Prevotella—Bacteroides*, *Veillonella*, and *Coriobacterium* groups [43].

The reinvestigation by FISH demonstrated that the apparent obviousness of clue cells detection by light microscopy is an illusion [43]. In many cases, the desquamated epithelial cells were not primarily coated, but secondary imprinted in otherwise independent bacterial accumulations as a consequence of mechanical laboratory manipulations. We called such seemingly covered cells "pseudo-clue cells" [43]. Likewise, illusory is the light microscopic impression, those bacteria with similar morphology are more or less evenly mixed all over the swab thus representing identical events. When resolved in constituting species, bacterial buildups within single swabs are often highly divergent and either the result of random mixture of various microbial groups in never repeating combinations (present in all samples) or structurally similar composed consortia (specific for each swab). Equalizing of thus different conditions is inadmissible.

Clue cells and "pseudo-clue cells" are principally different entities.

FISH demonstrates that characterization of complex polymicrobial microbiomes based solely on their density, morphology, and contact with epithelial cells (the only criteria assessable by light microscopy) is by no means sufficient [43]. The taxonomy-related characterization of polymicrobial complexes must above all assess the diverse intra-species relationships of bacteria belonging to the same species and the similarly versatile inter-species interactions between different bacterial species. The corresponding features which need to be considered for evaluation of polymicrobial vaginal microbiome are listed in Table 1.

Bacteria of the same taxonomy were distributed differently in each swab. Intra-species peculiarities in distribution of microbial groups can be well described by two features: **cohesiveness** to each other and **adherence** to epithelial cells. Accordingly, by combination of these two properties, four patterns of intra-species distribution can be distinguished: dispersed non-adherent bacteria (Figs. 1a and 2A), dispersed adherent bacteria (as example Fig. 1b), cohesive non-adherent bacteria (Fig. 2C), and cohesive adherent bacteria (Fig. 1B/c and 2D). The assessment of inter-species relations needs to document whether the particular species are distributed independently (autonomously) from each other or whether they are merged together constituting interlaced structures. The spatial association of interwind microbial groups may be occasional and observed in less than 20% of the glass slide surface or regular, involving a large portion of the sample. Bacterial conglomerates are usually unevenly distributed over the glass slide. The quantitative evaluation of permanent polymicrobial buildups is therefore more reliable, when related to the percent of epithelial cells rather than to the slide surface. Spatially organized structures observed in less than 20% of the sample cannot be assigned with certainty to regularly organized polymicrobial consortia.

By the joint evaluation of intra- and inter-species organization of the vaginal microbiome two forms of stable structural organization are most eye-catching: biofilms and sludges (Fig. 2C and D and 3A,B respectively). Biofilms need a surface on which they spread. Sludge is composed of self-sustained microbial accumulations floating in vaginal secretions without regular attachment to any surfaces. Bacteria within the matrix of such biofilm or sludge consortia are densely packed into a homogeneous mass with no free spaces between single participants. On the outer side of the consortia, bacteria tend to detach and their concentrations decrease as the distance to the core of consortium body increases and the gaps between single bacteria grow. Nevertheless, in our investigations the species-specific composition of consortia remains the same in consolidated and dissolved regions.

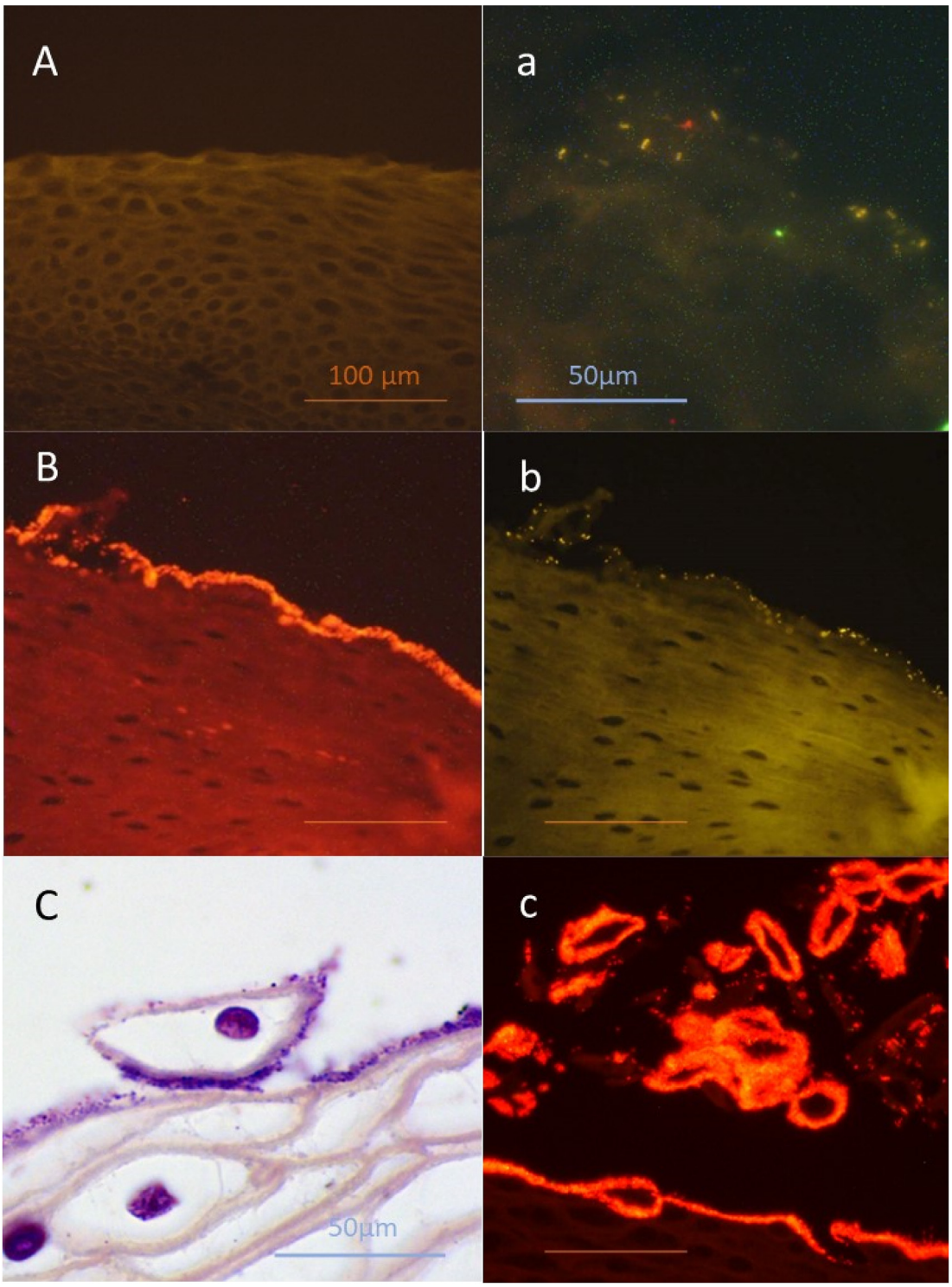

Fig. 1. Microphotographs of vaginal biopsies. Slices of biopsies were hybridized with a mix of two ribosomal RNA-targeted FISH probes, one specific for members of the genus *Gardnerella* (Gard662-Cy5 dark red fluorescence) and another specific for the genus *Lactobacillus* (Lab158-Cy3, yellow fluorescence). The 1A,a (x 400 left, x 1000 right side) panel demonstrates biopsy surface from healthy women. No bacteria are attached to the surface. In the region covered with discharge (1a), bacteria are detectable exclusively in slime and have no contact to the surface. Lactobacilli (in yellow) are predominant but also a single *Gardnerella* cell (red fluorescence) can be observed. The 1B,b panel demonstrates the surface of the biopsy from a woman with bacterial vaginosis (x400). The same microscopic field is presented on both sides. In dark red fluorescence (1B) a confluent *Gardnerella*-biofilm attached to the biopsy surface can be observed on the left. The yellow fluorescence on the right reveals that within the *Gardnerella*-biofilm abundant lactobacilli are present and tightly adherent to the vaginal epithelium. The 1C,c panel demonstrates that the desquamating epithelial cells are completely covered with biofilm prior to detachment, leading to formation of the clue cells in vaginal discharge (1c). The biofilm can be clearly seen using Gram stain (1C, x1000), but the species composition cannot be assessed in this way. All microscopic images presented in this manuscript are originals. They have been selected from a large number of photographs taken during previously performed and published studies [2,5,9,14,31,33] but were not included in the earlier manuscripts due to space limitations.

Table 1
Inter- and intra-species relations of vaginal polymicrobial complexes.

Inter-species relations / Intra-species relations		Species occurring autonomously or unrelated to other species	Interwind species		
			Locally or occasionally assembled on < 20% of the glass slide	Regularly interwind organized over ≥20% of the glass slide	
				Primarily frame-building	Secondary embedded
Cohesive	Adherent	n.o.	X	**Biofilm matrix** Fig. 1B/c,2D,3A	Consortia additive strains
	Non-adherent	n.o.	X	**Sludge matrix** Fig. 2C	Consortia additive strains Fig. 3a (arrow)
Disperse	Adherent	X Fig. 4A and partially 2B	X		Consortia additive strains Fig. 1b
	Non-adherent	X Fig. 2A	X		Consortia additive strains Fig. 1a

X: (not definitively structured) and erratically composed mixtures.
n.o.: observed with applied FISH probes, however, theoretically possible in case of mono-infections.

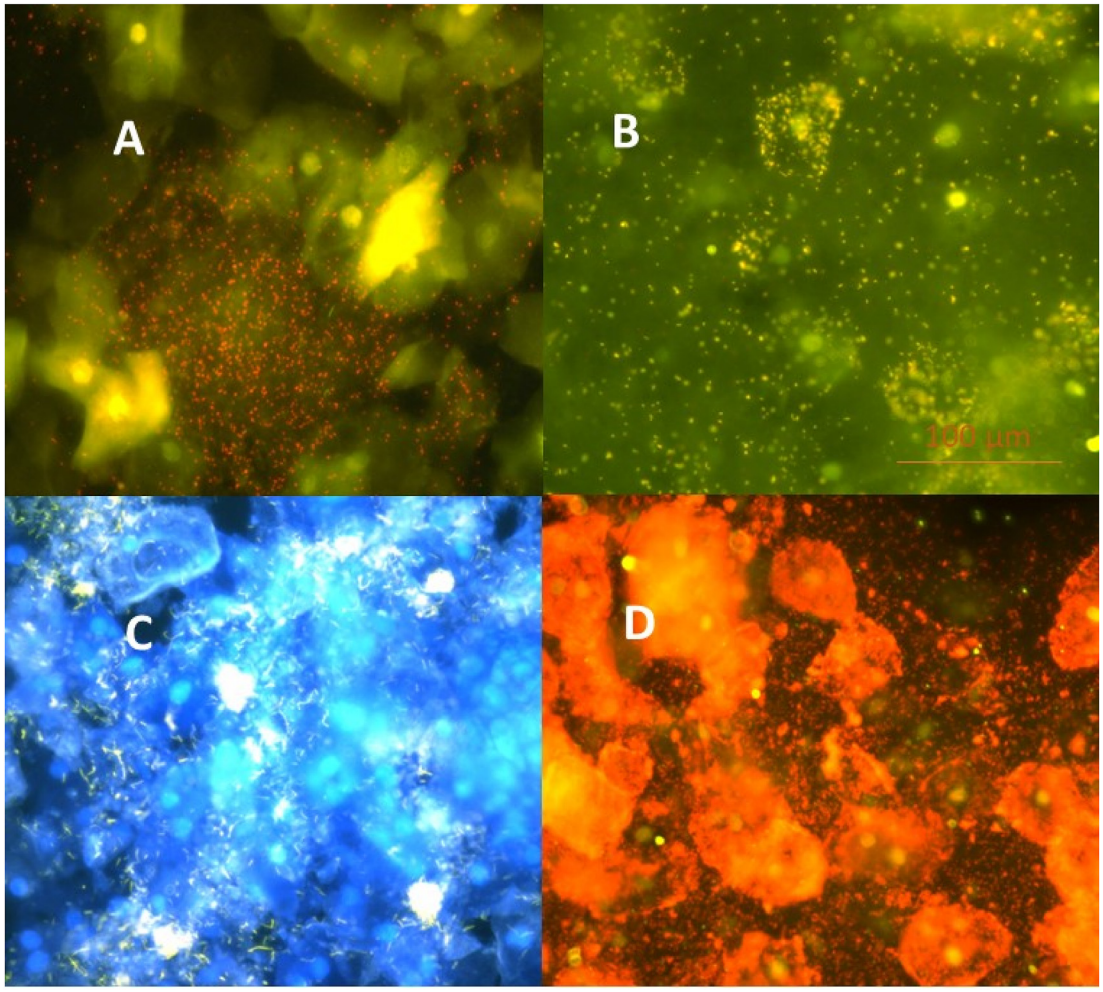

Fig. 2. FISH-images of vaginal smears from women diagnosed with bacterial vaginosis based on Amsel criteria. Panel 2A (*Enterobacteriaceae* specific Ebac1790-Cy5 probe, dark red fluorescence, x 400) demonstrates a dispersed non-adherent distribution of bacteria. Panel 2B shows dispersedly distributed *Lactobacillus iners* (Lin-Cy3, yellow fluorescence, x400) which are adherent to epithelial cells. Panel 2C demonstrates cohesive non-adherent sludge with matrix primarily constituted by *Lactobacillus iners* (Lin-Cy3, yellow fluorescence, counterstained with DAPI, those blue fluorescence highlights epithelial cells in the background). Panel 2D demonstrates a cohesive adherent mode of *Gardnerella*-biofilm attachment to clue cells (Gard662-Cy5 probe, dark red fluorescence, x 400) [14,43].

The unstructured mixes of bacteria can locally reach considerable concentrations and mimicking structured consortia. However, each of random agglomerations within the same smear has its own unique composition and the species-specific assemblance of condensed and dissolved regions is likewise dissimilar (Figs. 2 and 3) [43].

The presence of structured consortia in a sample does not mean that all species detected in the same swab are equally distributed in consortia and outside of them. Depending on involvement, bacterial species can be further divided into: i) constituting (primarily frame-building or secondarily embedded in the matrix, Fig. 3A–a); ii) indifferent (homogeneously distributed in and outside of the

consortium) or even iii) completely excluded from the biofilms and sludge matrixes or autonomous (Fig. 3B,b). The difference between constituting consortia species and autonomous microorganisms is easily perceptible. Species constituting consortia are localized mainly within biofilms or sludges, their concentrations outside the biofilm are markedly lower (Fig. 3A–a). The distribution of species uninvolved in consortia and autonomously spread is not synchronized, their concentrations outside of the consortia are markedly higher than within (Fig. 3B,b). Obviously embedded and autonomous species of the same taxonomy may have principally different qualities, with regard to forming of the consortia.

Not all species of the multiple species present in the BV microbiome are equally important for the consortium formation [43]. In all of the >8000 vaginal samples investigated to date, permanent framework building cohesive adherence (Figs. 1Bc, 2D and 3A) was found to be unique for *Gardnerella* species. The species building the framework of sludge could be either *Gardnerella* spp. or *L. iners* (and probably some close lactobacilli relatives). Apart from these two groups none of the other investigated species was able to build permanent contiguous-structured consortia in vaginal discharge. *F. vaginae*, low G + C (guanine + cytosine) bacteria other than lactobacilli, *Alpha-/Beta-/Gamma-/Delta-*

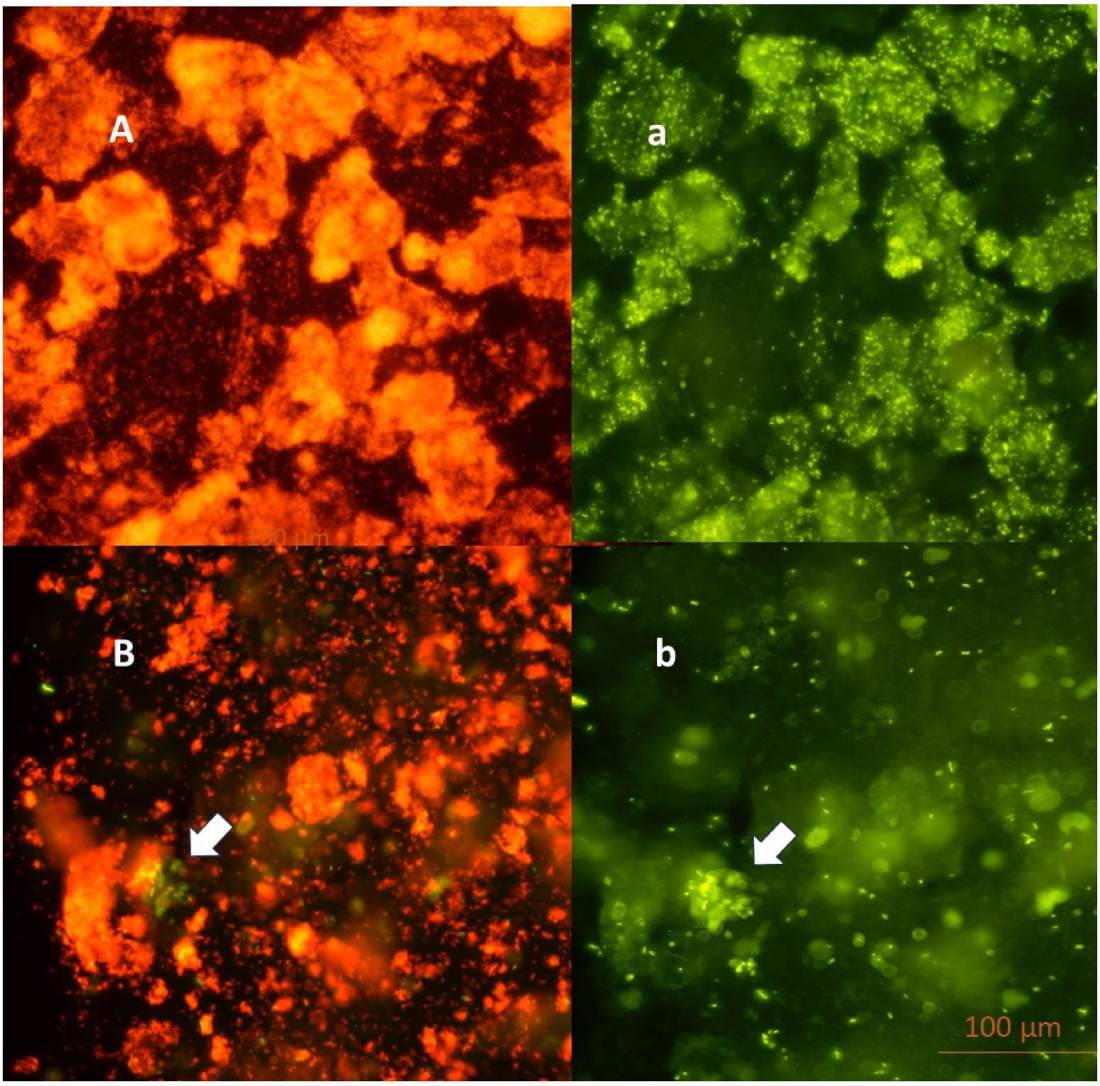

Fig. 3. Microphotographs of vaginal smears from women with bacterial vaginosis. BV was diagnosed based on Amsel criteria and samples hybridized with a mix of red stained *Gardnerella* (Gard662-Cy5) and yellow stained *Lactobacillus iners* (Lin-Cy3) FISH probes. Aa and Bb pairs demonstrate each the same microscopic field: *Gardnerella* spp. on the left and *Lactobacillus iners* on the right. The juxtaposing makes intra-species behavior of single and inter-species relations of both microbial groups obvious. From an intra-species point of view: In 3A, *Gardnerella* spp. is cohesive-adherent and builds biofilm around clue cells outlining them without any additional stain; in 3B, *Gardnerella* spp. is cohesive and forms a matrix of sludge, that is freely suspended in vaginal discharge and not attached to epithelial cells. The profiles of the epithelial cells are not seen in the dark red fluorescence of 3B panel as the bright luminescence of *Gardnerella*-sludge outshines them. However, the large round shadows of corresponding epithelial cells are well visible on the background fluorescence of panel 3b. *Lactobacillus iners* is in both examples mainly dispersed-distributed being adherent in 3a and non-adherent in 3b. In 3b *Lactobacillus iners* builds additionally an isolated cohesive non-adherent island (arrow). From an inter-specific point of view: *Gardnerella* spp. is in both examples primarily form-building of both types of consortia. *Lactobacillus iners* is interlaced and embedded in *Gardnerella*-biofilm. Concentrations of both microbial groups outside of the biofilm are markedly lower (3A,a). In 3Bb both bacterial groups are autonomously distributed. Neither their accumulations nor spread are related to each other spatially [43].

Proteobacteria, Enterobacteriaceae, *Acinetobacter, Helicobacter, Mycobacteria, Fusobacteria, Pseudomonas*, *Prevotella—Bacteroides*, *Cytophaga-Flavobacterium*, *Veillonella*, and *Coriobacterium* groups were, when present, involved exclusively as embedded (secondary interwind) constituents of *Gardnerella* spp. or *L. iners* primarily matrices or were distributed as dispersed independent and non-organized participants. Unfortunately, the detection of *Mycoplasma* spp. using 16/23S ribosomal gene-based FISH was unsuccessful in all our studies, probably due their absence of cell walls and thus we could not assess spatially the role of these very important microorganisms.

The most visually striking *Gardnerella*-biofilms were highly persistent and remained constant in repeated examinations for a duration of at least 12 weeks. In some cases, we could follow individual women with *Gardnerella*-biofilms for over 5—10 years, however systematic longitudinal studies are still required [41].

Except for *Gardnerella* spp. and *L. iners* consortia, the composition and availability of all other investigated species varied widely between single samples of the same patients and even more strikingly between different patients [5,41,43].

Although many questions remain open, it is obvious that BV diagnosed based primarily on light microscopy is prone to biases and therefore actually outdated. When light microscopy was compared with FISH, only 56% of smears with clue cells diagnosed in routine laboratories contained true clue cells covered with biofilm, while all other samples demonstrated pseudo-clue cells with epithelial cells mechanically embedded in sludge or in randomly assembled microbial masses. Depending on the gynecologic facility from which the samples originated, the proportion of pseudo-clue cells varied between 20 and 81% [43].

Structurally organized polymicrobial consortia are the most frequent pathologic entities and are easily recognizable by ribosomal FISH examination. However, polymicrobial interactions may not be restricted to consortia sharing microbial cell-to-cell contact. We previously demonstrated the presence of adherent urethra-vaginal *Escherichia coli* biofilms in women with vaginal discomfort after intercourse and “honeymoon cystitis” (Fig. 4A), as well as epithelia-invading *Candida* spp. overlapping with cohesive *Gardnerella* spp. and following subepithelial *Candida* spp. invasion without forming superficial biofilms or sludge (Fig. 4B,b) [44,45]. As such polymicrobial—host interactions are intraepithelial, these are not adequately represented in vaginal swabs. The clinical symptoms in many of these women overlapped broadly with those of BV, making the differentiation by means of the routine diagnostic tools (Amsel criteria, Nugent score, multiplex PCR) unreliable, especially in cases in which no biofilm forming *Gardnerella* spp. was co-involved [45]. Such variability in *Gardnerella*-biofilm formation is probably the consequence of varying virulence properties of different clusters among the species.

The species-specific ribosomal FISH identification and analysis of microbial distribution in BV suggests that bacterial vaginosis is a complex syndrome and an umbrella term of a number of mostly different polymicrobial infections assembled by multiple species and only seemingly sharing similar clinical symptoms. As long as these infections are not reliably and specifically diagnosed, studies on BV epidemiology, therapy response, partner treatment, sexual transmission, persistence and recurrence will inevitably lead to variable and controversial results, depending on the real composition of the individual patient groups.

The situation will become clearer when different consortia are specifically studied and the fuzzy nosology of bacterial vaginosis is subdivided according to its specific etiology. It is currently impossible to predict how many of these specific diseases will emerge in the end. We reviewed the data on clinical and epidemiologic implications of *Gardnerella*-biofilms previously and will only shortly mention them here. These data demonstrated, that biofilm-vaginosis or uro-genital *Gardnerella*-biofilm is probably a better name for the condition and it represents the dominant pathology within the BV syndrome. Biofilm vaginosis involves both females and males and is sexually transmitted through the biofilm-covered clue cells, suspended in vaginal/preputial secretions or sperm [46] and hence cause transfer of intact biofilm from partner to partner. Such biofilm can extend to the distal urethra and even colonize the endometrium leading to BV associated pelvic or obstetric complications including infertility [41,47]. The tight adherence of *Gardnerella*-biofilm to the epithelial layer impacts the hosts native immunity and facilitates acquisition of classical sexual transmitted infections. Microbial multispecies cooperation within biofilm helps it to resist antimicrobials making recurrence of symptomatic BV a major therapeutic challenge [15,48—51].

Unfortunately, ribosomal FISH technology, which directly and uniquely investigates the spatial organization of polymicrobial consortia is presently performed and available only in very few laboratories and thus not available for clinical routine use or broad scale research. In contrast, currently available PCR and NGS methods have reached a level of perfection, automation and availability that allows them to be used everywhere with consistent quality. However, while both PCR and NGS can accurately identify and list the participants within polymicrobial mixes, they are unable to directly visualize their morphology or spatial organization in determining their pathogenic role in BV. Yet, since any morphology needs a specific genetic script, finding of PCR/NGS gen-patterns and criteria, which correspond to each of the pathogenic polymicrobial consortia is only a question of further additional deliberate research. The correlation of FISH with PCR data and full genome sequencing will then open a new perspective in validation and refinement of polymicrobial infectiology.

5. Conclusions for updating the infection theory

The human body constantly encounters bacteria from the outer world. The regular presence or absence of microorganisms is regarded as the norm, while their appearance or disappearance when associated with symptoms and morphologic changes, is seen as a disorder. In medical textbooks, the regular predictable colonization of the body surfaces is called eubiosis, and its shift is named dysbiosis. Detection of specific microorganisms in various anatomic sites or tissues, where they are usually absent, makes them suspects for the causation of local pathology. However, only tracing the chain of infection or experimental initiation of disease by suspected pathogens confirms the critical causal interrelationship [52]. Such confirmation may be extremely difficult, but is indispensable. Following this route of research in infectious diseases, stunning pathways such as in malaria or pest were previously demonstrated [53]. Recognition of *de novo* infections in heavily colonized regions, although likewise successful, was however mainly restricted to scenarios whereby normally the pathogens are absent and remained inconclusive when bacteria are found both in health and disease states [52]. Reverse conclusions, in which the disappearance of bacteria typical for eubiosis could be responsible for dysbiosis are rarely established. Still, they remain widespread, probably, since finding of a specific feature is critical. The lack of clinical or laboratory specificity rules out establishing causality early in the search for exact pathogenetic mechanisms.

However, the desired specificity is not inevitably restricted to single microorganisms. Duets, triplets, quartets and higher organized groupings of microorganisms including sludge and biofilms may play a role as specific infectious agents [1,5,41,43,44]. Each component of such consortia may be harmless when occurring on its own and may be found in many unrelated situations. However,

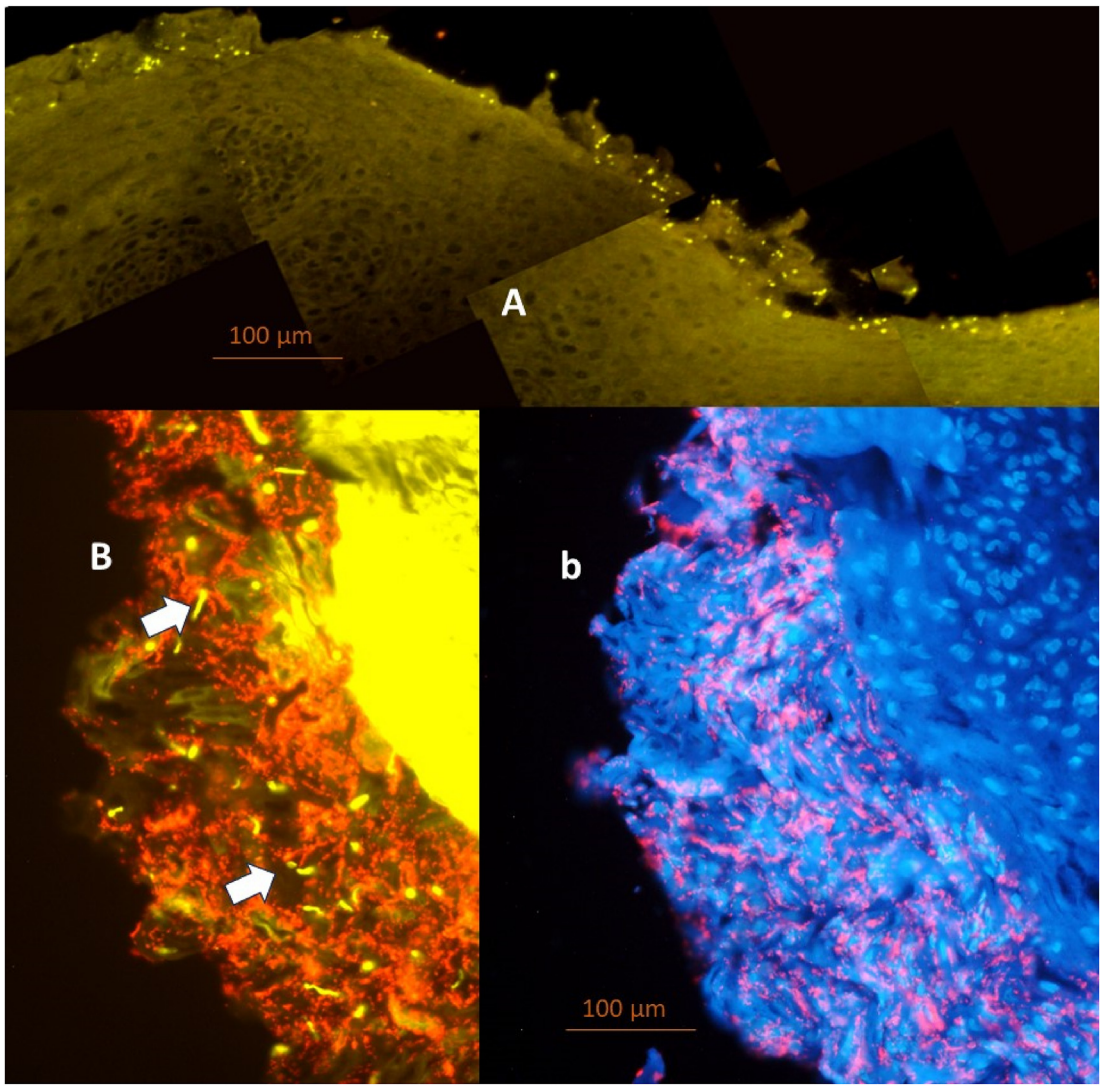

Fig. 4. FISH images of the vaginal biopsies from women with postcoital cystitis (A) and candidiasis (B,b). Panel 4A demonstrates a yellow fluorescence of *Escherichia coli* which is adherent to the vaginal epithelium in a dispersed manner. The biopsy was taken from a woman with recurrent postcoital cystitis complaints (Ebac1790-Cy3 probe). Panels 4B and 4b demonstrate a multicolor hybridization with yellow stained *Candida* spp. (Caal-Cy3) and red *Gardnerella* spp. (Gard662-Cy5) probes, and counterstained with unspecific blue fluorescent DAPI stain, which binds DNA. Thick yellow hyphae of *Candida* spp. appearing under the microscope as "rods" (arrows) are located between prolific and cohesive red-fluorescent *Gardnerella* spp. agglomerates. Contrasting of *Gardnerella* spp. against DAPI makes obvious, that in this particular case, *Gardnerella* spp. are not forming adherent biofilm tightly attached to the epithelial surface (the outer vaginal surface is completely free of biofilm), but is co-invading the vaginal wall. The interlacement of *Gardnerella* spp. and *Candida* spp. in Fig. 4B is spurious. Viewed closely under microscope, *Candida* spp. is located between epithelial cells of the invaded vaginal epithelium and not directly contacting *Gardnerella* spp. (4B), while *Gardnerella spp.* grows squeezed in epithelial lacunas "pre-broken" by *Candida* spp. (4b) [44,45]. (In 2D images, microorganisms from separate layers sometimes project onto each other, simulating contact of *Candida* with *Gardnerella* spp. cells, which is not confirmed when resorting to 3D microscopy). The bright yellow signal on the right side of Fig. 4B is unspecific background noise due to overexposure of background fluorescence after superimposition of different multicolor channels.

as soon as assembled, pathogenic collaboration enables consortia to grow and prevail under conditions in which each of participants would inevitably perish.

Historically, infectious disease research has focused on individual pathogens, their specific appearance and distribution within the body being easier to track. Polymicrobial involvement was invariably considered as secondary and mostly neglected. The mono-infectious approach was sufficient and probably unavoidable as long as microbial culture isolation was the only way to detect bacteria. With the increase of availability of culture independent molecular-genetic methods the previously seemingly clear differentiation between mono- and polymicrobial involvement especially in heavily colonized regions became practically impossible. Too many independent players could be detected in the same sample. In addition, many of the participants are present in both health and disease. In our opinion, declaring only one of them relevant and all other secondary, is no longer plausible. However, the step from the mono-infectious perspective to the pathogenic consortia has not been taken yet. The concept of polymicrobial infections caused by stable microbial communities has not entered into common clinical thinking. Microbiomes are further viewed by the medical majority only in terms of singularity of independently assembled microorganisms.

Many commercial laboratories at present offer extended analysis of individual patient microbiomes at more or less acceptable prices. Different kinds of composition scores, alpha- and beta-

diversities, and similar disjointed groupings are proposed but fail to achieve any clinically relevant progress. Unfortunately, in such studies the possible cooperation ties and joint pathogenic actions remain neglected. The large amount of detected microorganism sequences is impressive, but in reality, the excess of disjointed data oftentimes only confuses. The diagnostic value of the new scientific findings revealed does not exceed the lapidary statement – "something is wrong, you should ask your doctor". This is not surprising. Laboratory microbiome reports to physicians in terms of occurrence and quantity of participants say nothing about the interrelationships of single microorganisms and potentially leads to iatrogeny. In analogy, even the most exact listing of objects on the night sky, detected by automated telescope and presented in numeric clusters, tells nothing about the structure of the universe and causal interrelationships of its components. The mono-infectious approach and thinking should be specifically extended to polymicrobial involvement. Malaria is not possible without mosquitos, as epidemic typhus and pest (*Yersinia pestis*) cannot happen without lice, and fleas and rats, respectively [53]. Why should be microorganisms in complex environment relying only on themselves? We know in the meantime from *in vitro* experiments that polymicrobial interactions may be manifold and highly sophisticated, including quorum sensing, multiple biochemical, antigenic and virulence properties shared and complementing each other [51]. The host is not passive either, its surfaces resist unwanted colonization. To be durable, pathogenic consortia must be able to prevent their replacement from the colonized niche, they must be able to adhere to the host surfaces and persist while simultaneously maintaining their composition [5,41]. An essential sign of such capacities in the vaginal environment is a verifiable cohesiveness of the crucial participants to each other and their adherence to living or at least to desquamated epithelial cells of the host. With regard to BV, only two microbial groups: *Gardnerella* spp., and *L. iners* build cohesive sludge in a subset of patients and only the former can definitively adhere and enwrap epithelial cells nearly completely yet covering the vaginal epithelium with polymicrobial biofilms [43]. The capability of durable cohesiveness and biofilm building must be dependent on specific species and genes responsible for these properties. It is time to identify these species and genes developing PCR and NGS based tools, which instead of only passive listing of occurrences, can track the suspect polymicrobial consortia within individual microbiomes, bringing simple, reliable and convincing criteria of polymicrobial infections into clinical practice. Broad scale clinical investigations will allow then to follow the roles of biofilms and cohesive sludge in clinical outcome of presently blurry syndromes such as BV.

CRediT authorship contribution statement

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Declaration of competing interest

The authors have no conflict of interest (including financial commitments or ethical concerns). The data of this publication were not presented previously. The work has not been published previously and is not under consideration for publication elsewhere. No generative artificial intelligence was used. The publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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