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Gardnerella vaginalis outcompetes 29 other bacterial species isolated from BV patients in an *in vitro* biofilm formation model

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Abstract

Despite the worldwide prevalence of bacterial vaginosis (BV), its etiology is still unknown. Although BV has been associated with the presence of biofilm, the ability of BV-associated bacteria to form biofilms is still largely unknown. Here, we isolated 30 BV-associated species and characterized their virulence, using an *in vitro* biofilm model. Our data suggests that *G. vaginalis* had the highest virulence potential, as defined by higher initial adhesion and cytotoxicity of epithelial cells, as well as the greater propensity to form a biofilm. Interestingly, we also demonstrated that most of the BV-associated bacteria had a tendency to grown as biofilms.

Key words: Bacterial vaginosis, multi-species biofilm, virulence determination

Introduction

Bacterial vaginosis (BV) is one of the most common vaginal disorders of women, of reproductive age; it has been associated to an abnormal pregnancy, inflammatory pelvic disease and increased risk of acquiring sexual transmitted diseases [1]. The high prevalence, high relapse rate, and associated complications, make BV of paramount importance. Yet, despite its impact on womens health, little is known about the pathogenesis of BV. Due to the detection of multiple bacterial species in BV patients, many researchers consider it a polymicrobial infection [2]. Importantly, Swidsinski *et al.* first reported the presence of a multi-species biofilm, mainly formed by *Gardnerella vaginalis,* in the vaginal epithelium of women with BV [3]. However, while some attention is now focused on *G. vaginalis* biofilms, the role of the other BV-associated bacteria has been somewhat neglected. To better understand BV etiology, the isolation and culture of BV-associated bacteria is necessary, in order to determine their virulence potential.
We recently demonstrated that, despite having a lower ability to adhere and displace lactobacilli from an inert surface, *Atopobium vaginae* and *Fusobacterium nucleatum* were able to stimulate and enhance biofilm formation by *G. vaginalis* [4]. This suggested that some species might have an important role in BV as secondary pathogens. However, current knowledge about the specific role of most BV-associated bacteria is still scarce since functional microbiological studies often use very limited number of species [5]. Here, in a first approach to determine the role of BV-associated bacteria in biofilm formation, we isolated 30 bacterial species, from BV patients, and evaluate their virulence potential using an *in vitro* biofilm model. We characterized their ability to adhere to epithelial cells and to induce cytotoxic changes, as well as to grow as biofilms. Their susceptibility, to antibiotics commonly used in BV treatment, was also determined.

Methods

Vaginal Samples, Bacteria Isolation and Identification

Vaginal samples were obtained from volunteer women after informed consent as approved by the Minho University Institutional Review Board (SECVS 003-2013). Samples were analyzed by microscopy after Gram staining and BV diagnosis was based on Nugent criteria [6]. BV positive vaginal samples were used for bacteria isolation in anaerobic conditions as previously described [7]. Briefly, bacteria were isolated using Columbia Agar supplemented with 5% horse blood (CBA) and incubated at 37°C in the presence of 5%, 10% CO_2 or in anaerobic conditions generated by AnaeroGenTM (Oxoid). Plates were incubated for up to one week. Where

possible, bacteria were identified by DNA sequencing of the *16S* rRNA gene. However, due to high homology in *Staphylococcus* spp and *Corynebacterium* spp, their identification was performed by DNA sequencing of *rpoB* gene. Additionally, biochemical tests were also performed for organisms where DNA sequencing could not identify the isolate. More details are listed in supplementary table 1.

Initial Adhesion to Epithelial Cells and Cytotoxicity Assays

Initial adhesion to epithelial cells was performed as previously optimized [8]. Briefly, bacterial suspensions, grown in supplemented brain heart infusion broth (sBHI) containing 2% (w/w) gelatin, 0.5% yeast extract, 0.1% starch and 0.1% glucose, were resuspended in DMEM medium to 1×10^8 CFU.mL^{-1} and were added to a monolayer of HeLa cells, for 30 min at 37ºC, in anaerobic conditions. After washing the non-adherent bacteria, cells were fixed with methanol and adhesion was microscopically quantified and expressed as the average number of bacteria per epithelial cell. Cytotoxicity assays were performed with a bacterial concentration of 2.9×10^7 CFU.mL^{-1} with an incubation period of 3 H [5]. Cytotoxicity was scored as follows: 0, no difference between the test and the control; 1, 25% of the cells were rounded; 2, 25–50% of the cells were rounded; 3, 50% of the cells were rounded; 4, 50% cells were rounded, with partial disruption of the monolayer and 5, complete disruption/absence of the monolayer. Twenty fields of view were acquired per experiment and three independent experiments, with technical duplicates, were performed for each analysis.

Biofilm Formation and Quantification

Biofilms were grown in 9 different culture media: Luria broth (LB); LBG (LB supplemented with 0.25% (w/v) glucose); de Man-Rogosa and Sharpe agar (MRS); MRSG; Tryptic Soy broth (TSB); TSBG; sBHI (BHI supplemented with 2% (w/w) gelatin, 0.5% (w/w) yeast extract, and 0.1% (w/w) starch); sBHIG and sBHIF (sBHI supplemented with 10% (v/v) fetal bovine serum). Biofilms were qualitatively quantified with safranin staining [5]. Subsequently, the intrinsic ability to form biofilms was quantified as described by Harwich *et al*. [9] using the 3 media that promoted the greatest biofilm growth, ensuring that one was common to all isolates. The biofilm formation index (BFI) was defined as the average number of bacteria grown as biofilms, in the 3 selected media. The data analysis was based on at least 3 independent experiments.

Antibiotic Susceptibility

The susceptibility of the isolated bacteria to the 3 most common antibiotics used for BV treatment, namely metronidazole (MD), tinidazole (TZ) and clindamycin (CM), was evaluated by determining the minimum inhibitory concentration (MIC).

Statistical Analysis

Independent unpaired data were analyzed using student's t-test and statistical package for the social sciences (SPSS) software (IBM, Chicago, IL, USA). A p-value less than 0.05 was considered significant.

Results

Vaginal samples were obtained from 40 Portuguese women, between 18 and 40 years old. BV was found in 11 women and these samples were selected for bacterial isolation. 30 distinct bacterial species were isolated and 1 strain, per species, was selected for further studies (table 1).

Table 1 | Initial adhesion, cytotoxicity score and biofilm formation index (BFI) of the vaginal bacteria isolated from Portuguese women diagnosed with BV. Experiments were repeated three times.

Bacteria	Bacteria/HeLa cell (Mean ± SD)	Cytotoxicity score [a]	BFI [b] (Mean ± SD)	Antibiotics – MIC[c] range (µg.mL^{-1})		
				MD	TZ	CM
Gardnerella vaginalis	13.54 ± 2.83	4	87.60 ± 6.26	> 128	32 - 64	< 0.01
Streptococcus agalactiae	2.01 ± 0.49	3	53.36 ± 12.64	> 128	> 128	> 128
Streptococcus anginosus	1.74 ± 0.30	3	64.90 ± 20.13	> 128	> 128	> 128
Staphylococcus epidermidis	1.18 ± 0.12	3	49.30 ± 12.79	> 128	> 128	> 128
Shigella spp	1.06 ± 0.19	3	27.71 ± 6.64	> 128	> 128	> 128
Staphylococcus haemolyticus	0.71 ± 0.06	3	44.30 ± 7.00	> 128	> 128	0.125 – 0.06
Klebsiella/Enterobacter	4.24 ± 0.39	2	25.17 ± 4.72	> 128	> 128	> 128
Corynebacterium tuscaniense	1.77 ± 0.34	2	82.34 ± 8.78	> 128	> 128	> 128
Corynebacterium amycolatum	1.64 ± 0.37	2	71.22 ± 17.52	> 128	> 128	> 128
Aerococcus christensenii	1.51 ± 0.08	2	74.27 ± 14.18	> 128	> 128	0.01 – 0.06
Staphylococcus hominis	1.21 ± 0.12	2	80.27 ± 11.62	> 128	> 128	> 128
Actinomyces turicensis	1.18 ± 0.00	2	80.80 ± 5.49	> 128	> 128	< 0.01
Lactobacillus vaginalis	0.95 ± 0.10	2	48.59 ± 8.27	> 128	> 128	2 -4
Bifidobacterium longum	0.84 ± 0.15	2	54.67 ± 7.28	> 128	> 128	< 0.01
Staphylococcus fleuretti	0.84 ± 0.20	2	57.84 ± 12.23	> 128	> 128	4
Gemella haemolysans	0.80 ± 0.43	2	53.51 ± 3.70	> 128	> 128	> 128
Bacillus firmus	0.78 ± 0.23	2	23.91 ± 5.20	> 128	> 128	64 - 128
Actinomyces neuii	0.65 ± 0.10	2	72.42 ± 9.18	> 128	> 128	0.25 – 0.125
Enterococcus faecalis	0.42 ± 0.06	2	78.91 ± 10.50	> 128	> 128	64 – 128
Brevibacterium ravenspurgense	0.36 ± 0.04	2	52.71 ± 12.75	> 128	> 128	> 128
Propionibacterium acnes	0.12 ± 0.08	2	58.56 ± 6.47	> 128	> 128	0.01 – 0.06
Mycoplasma hominis	12.60 ± 5.43	1	73.84 ± 21.01	> 128	> 128	0.125
Corynebacterium tuberculostearicum	5.78 ± 0.69	1	56.52 ± 6.29	> 128	> 128	64 - 128
Staphylococcus warnerii	5.16 ± 0.29	1	50.08 ± 11.09	> 128	> 128	0.25
Staphylococcus saprophyticus	4.35 ± 0.72	1	59.50 ± 24.31	> 128	> 128	> 128
Nosocomiicoccus ampullae	3.80 ± 0.07	1	69.23 ± 7.79	> 128	> 128	4
Staphylococcus simulans	1.81 ± 0.02	1	64.73 ± 10.89	> 128	> 128	> 128
Escherichia coli	1.20 ± 0.12	1	23.56 ± 6,96	> 128	> 128	> 128
Breviobacterium mcbrellneri	0.56 ± 0.14	1	75.09 ± 7.67	> 128	> 128	64 - 128
Micrococcus spp	0.16 ± 0.09	1	65.65 ± 6.02	> 128	> 128	0.06 – 0.25

Note. [a] Cytotoxicity was scored as follows: 0, no difference between the experimental well and the control; 1, < 25 % cells were rounded; 2, 25-50 % cells were rounded; 3, >50 % cells were rounded; 4, > 50% were rounded, with partial disruption of the monolayer; 5, complete disruption/absence of the monolayer

[b] The index of intrinsic capacity for biofilm formation was calculated using the average of the 3 growth media with higher biofilm growth, including a common media.

[c] MIC, Minimum inhibitory concentration.

Some of the isolated species had never been associated with BV cases before, such as *Brevibacterium ravenspurgence*, *Corynebacterium tuscaniense*, *Nosocomiicoccus ampullae*, *Staphylococcus warneri*, *Staphylococcus fleuretti* and *Bacillus firmus* (supplementary table 1).

We first quantified the ability of the selected isolates to adhere to a monolayer of HeLa epithelial cells. Interestingly, there were dramatic differences in adhesion between *G. vaginalis* and *Mycoplasma hominis* relative to the remaining BV isolates, with *G. vaginalis* demonstrating higher initial adhesion ability. The cytotoxic effect on these epithelial cells was also evaluated,

since it is an important trait in BV [5]. Of note, we observed that the majority of bacteria (80%) had very low cytotoxicity, with *G. vaginalis* showing the highest cytotoxicity score.

In order to determine the optimal medium, for *in vitro* biofilm formation, all isolates were initially cultured anaerobically, using 9 different growth media. As expected, our qualitative analysis showed different biofilm-forming capability, by the different species, and this was highly dependent on the media used (supplementary table 2). Hence, to quantitatively determine each species tendency to grow as biofilm, we determined the BFI. Our results showed that *G. vaginalis* was the bacteria with highest BFI, when compared to the others isolates. Surprisingly, our results also demonstrated that most BV-associated bacteria, such as *M. hominis*, *Staphylococcus hominis*, *Brevibacterium mcbrellneri* and *Enterococcus faecalis,* also showed significant biofilm-forming capability.

Finally*, in vitro* susceptibility of isolates to MD, TZ and CM was evaluated by determining the MIC. All bacteria tested were resistant to MD and TZ. Conversely, the number of bacteria resistant to CM was only 67%. Our results, were not surprising since an increasing incidence of resistance associated with conventional antibiotic therapy, for species that have been associated with BV, has previously been demonstrated [10].

Discussion

BV is often considered a polymicrobial infection due to the frequent identification of multiple bacterial species associated with this condition. However, the limitations associated with classical microbiological culture techniques have inadvertently affected the ability of scientists to understand the etiology of this clinical condition. As a polymicrobial infection, different species might have different roles in the initiation and progression of BV. Since BV is associated with biofilm formation, it has been proposed that *G. vaginalis* might compete with vaginal *Lactobacillus* spp. and enable other BV-associated bacteria to be incorporated and grow within the biofilm [5]. Theoretically, an early colonizer would contribute to initial adhesion to the epithelium, while late and/or secondary colonizers could contribute to biofilm maturation. This is a process that has been extensively studied in oral biofilms [11] and we recently demonstrated this principle, in BV, by using mixed biofilms of *G. vaginalis*, *Mobiluncus mulieris*, *A. vaginae*, *Prevotella bivia* and *F. nucleatum* [4]. Being the most prevalent bacterial species of BV-associated biofilms, most studies have tended to focus on *G. vaginalis*. To highlight its contribution to pathogenicity, biofilm formation has already been shown to increase tolerance of *G. vaginalis* to external stresses [12], including tolerance to antibiotics, with implications for high recurrence and relapse rates in those persons predisposed to BV [13]. Prior to this work,very few studies have investigated biofilm formation of other BV-associated bacteria [1]. To better understand the virulence potential of the multiple species found in BV, we used an *in vitro* biofilm model, and tested the virulence potential of 30 different species isolated from BV patients.

Since initial adhesion is the first step in biofilm formation [14], we first aimed to assess which species were likely to be most prevalent in the early stages of BV development. Interestingly, only *G. vaginalis* and *M. hominis* adhered avidly to HeLa cells, suggesting that these two species could play an important role as early colonizers in BV. However, *M. hominis* revealed very low cytotoxicity to HeLa cells, contrary to *G. vaginalis.* Furthermore, an important aspect that undermines the possible role of *M. hominis* as the main agent of BV is related to the relative prevalence of both species in BV, since *M. hominis* is found less often than *G. vaginalis* [15]. All other strains had very low adhesion and cytotoxicity levels. Interestingly, we did not find any correlation between initial adhesion and cytotoxicity, suggesting that cytotoxicity is not an entirely density dependent process.

We then determined the intrinsic capability for biofilm formation, as described first by Harwich *et al*. [9]. Since we were unable to identify a single growth media that was optimal for all species, we defined the BFI as the average of bacterial growth as biofilm, using 3 distinct growth media. With this approach, we tried to minimize the limitations of *in vitro* biofilm formation, since not all factors found *in vivo,* are reproducible *in vitro*. Despite this limitation, only 3 species showed lower ability to grow as biofilm (BFI < 30%) in contrast to all the remaining tested species.

Finally, we detected high levels of resistance against common antibiotics used to treat BV, in all isolated species. Bacteria were more susceptible to CM than to MD or TZ, which was expected, based on previous studies that reported a high resistance to metronidazole [10]. Without an identified etiological agent, BV treatment has been based on empiricism, which can contribute to the increase in antimicrobial resistance [14]. Importantly, high antibiotic resistance acquisition rates have been associated with multi-species biofilms [14], and this could explain the similar and high resistance levels found in our study.

In conclusion, this study sheds new light on the possible roles of many bacteria associated with BV and, for the first time, directly compares their biofilm forming ability. Even though our data provides strong evidence that *G. vaginalis* could be the main colonizer in BV, our results also demonstrated that other BV-associated species have a high capacity to grow as biofilm. Yet to be determined is whether these bacteria opportunistically join the mixed-species biofilms, often found in BV, with no consequence to the outcome of the disease, or if these species can interact synergistically with *G. vaginalis,* as shown before for *A. vaginae* and *F. nucleatum,* enhancing biofilm forming ability, and therefore contributing to the high relapse rates found in BV.

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Supplements

Supplementary Table 1 | Identification of BV isolates. The partial sequences of 16S rRNA or rpoB coding genes obtained after DNA sequencing were analysed using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for bacteria identification.

Bacteria	Gene	Sequence Length	Query Coverage (%)	Maximum Identity (%)	Accession Number	Biochemical tests*
Actinomyces neuii UM067	16S rRNA	740	99	99	NR_042429.1; NR_042428.1	G, +/-
Actinomyces turicensis UM066	16S rRNA	734	99	99	NR_037020.1	G -/-
Aerococcus christensenii UM137	16S rRNA	735	99	99	NR_044929.1	G -/-
Bacillus firmus UM034	16S rRNA	699	100	98	NR_025842.1	G
Bifidobacterium longum UM062	16S rRNA	702	100	99	NR_043437.1; NR_074744.1	G -/-
Brevibacterium ravenspurgense UM066	16S rRNA	722	99	99	NR 044398.1	G +/+
Brevibacterium spp UM137	16S rRNA	722	99	98	NR_026299.1; NR_025375.1	G +/+
Corynebacterium amycolatum UM065	rpoB	384	98	99	HE586300.1; AY492241.1;HE586299.1	G +/-
Corynebacterium tuberculostearicum UM137	rpoB	398	98	94	AY581869,1	G +/-
Corynebacterium tuscaniense UM137	rpoB	400	95	94	FJ416377.1	G +/-
Enterococcus faecalis UM035	16S rRNA	745	100	99	NR_074637.1; NR_040789.1	G
Escherichia coli UM056	16S rRNA	723	99	99	NR_026331.1; NR_026332.1; NR_027549.1; NR_024570.1	g, +/-, L, I, LMX
Gardnerella vaginalis UM224	16S rRNA	723	99	99	NR_074227.1; NR_044694.1	Gg, -/-
Gemella haemolysans UM034	16S rRNA	732	99	99	NR_025903.1	G
Klebsiella/Enterobacter UM062	16S rRNA	740	99	99	NR_074729.1; NR_025635.1; NR_036794.1; NR_037084.1; NR_074913.1; NR_041750.1; NR_024643.1; NR_024640.1	g, +/-
Lactobacillus vaginalis UM062	16S rRNA	760	99	99	NR 041796.1	G, -/-
Micrococcus spp UM067	16S rRNA	712	99	99	NR 075062.1; NR 037113.1; NR 044365.1	G, +/-
Mycoplasma hominis UM054	16S rRNA	733	99	99	NR_041881.1	g
Nosocomiicoccus ampullae UM121	16S rRNA	629	99	99	NR 044444.1	G, +/+
Propionibacterium acnes UM034	16S rRNA	725	99	99	NR_074675.1; NR_040847.1	G

Shigella spp UM137	16S rRNA	743	99	99	NR_074894.1; NR_026331.1; NR_027549.1; NR_074902.1; NR_026332.1; NR_074892.1; NR_074893.1; NR_074891.1; NR_074882.1; NR_024570.1	g, +/-, L, I, lmx
Sreptococcus agalactiae UM035	16S rRNA	329	98	99	NR_040821.1; NR_036918.1; NR_037101.1	G
Staphylococcus epidermidis UM066	rpoB	845	99	98	CP000029.1;AE015929.1	G, +/-
Staphylococcus fleuretti UM121	rpoB	780	95	90	GC222236.1	G, +/+
Staphylococcus haemolyticus UM066	rpoB	847	99	100	AP006716.1	G, +/-
Staphylococcus hominis UM224	rpoB	679	80	98	EF173661.1	G, +/-
Staphylococcus saprophyticus UM121	rpoB	832	100	99	AP008934.1	G, +/-
Staphylococcus simulans UM059	rpoB	755	99	99	NR 036906.1	G, +/-
Staphylococcus warnerii UM224	rpoB	488	99	100	AF325895.1	G, +/-
Streptococcus anginosus UM241	16S rRNA	756	99	99	NR 041722.1	G, -/-

*Results obtained for the biochemical tested performed: Gram-positive (G), Gram-negative (g), catalase and oxidase-positive (+/+), catalase and oxidase- negative (-/-), catalase-positive and oxidase-negative (+/-), lactose fermentation positive (L), indole-positive (I), fluorescence under UV light of LMX medium (LMX), absence of fluorescence under UV light of LMX medium (lmx).

Supplementary Table 2 | Qualitative analysis of biofilm-forming ability of bacterial vaginosis isolates. The ability of isolates to form biofilm was tested in 9 different growth media commonly used in biofilm formation assays.

	Media								
Strains	**LB**	**LBG**	**MRS**	**MRSG**	**TSB**	**TSBG**	**sBHI**	**sBHIG**	**sBHIF**
Actinomyces neuii UM067	+-	+-	+-	+-	+-	+++	+-	+-	+-
Actinomyces turicensis UM066	-	-	-	++	++	+++	++	++	+-
Aerococcus christensenii UM137	-	+-	-	-	-	+++	++	+-	+-
Bacillus firmus UM034	+-	+-	+-	+-	+-	+-	+-	+-	+-
Bifidobacterium longum UM062	+-	+-	+-	+-	+-	+-	+-	+-	++
Brevibacterium ravenspurgense UM066	+-	+-	-	-	-	-	++	+-	+-
Brevibacterium mcbrellneri UM137	++	++	++	++	++	+++	+++	++	+-
Corynebacterium tuscaniense UM137	+-	+-	++	+-	++	+++	++	++	+-
Corynebacterium amycolatum UM065	+-	+-	+-	+-	+-	+-	++	++	+-
Corynebacterium tuberculostearicum UM137	+-	+-	+-	+-	+-	+-	+-	+-	+-
Enterococcus faecalis UM035	+-	++	+-	+-	+-	++	++	+-	+-
Escherichia coli UM056	+-	+-	+-	+-	+-	+-	+-	+-	+-
Gardnerella vaginalis UM224	-	-	+-	+-	+-	+-	++	+++	++
Gemella haemolysans UM034	+-	+-	+-	+-	+-	+-	+-	+-	+-
Klebsiella/Enterobacter UM062	+-	+-	+-	++	+-	+-	+-	+-	+-
Lactobacillus vaginalis UM062	-	-	-	-	-	-	+-	+-	+-
Micrococcus spp. UM067	+-	+-	+-	+-	+-	++	++	++	+-
Mycoplasma hominis UM054	-	+-	-	-	-	+++	++	+-	+-
Nosocomiicoccus ampullae UM121	+-	+-	+-	+-	++	++	++	++	++
Propionibacterium acnes UM034	++	++	-	-	-	-	++	+-	++
Streptococcus agalactiae UM035	-	-	-	-	++	++	+-	++	++
Shigella spp UM137	+-	+-	+-	+-	+-	+-	+-	+-	+-
Staphylococcus epidermidis UM066	+-	++	++	++	++	++	+-	++	++
Staphylococcus fleuretti UM121	+-	+-	+-	++	+-	+-	+-	++	+-
Staphylococcus haemolyticus UM066	+-	+-	+-	+-	++	+-	++	+-	++
Staphylococcus hominis UM224	+-	+-	+-	++	+-	++	++	++	+-
Staphylococcus simulans UM059	+-	+-	+-	+-	+-	++	+-	+-	+-
Staphylococcus saprophyticus UM121	+-	+-	+-	+-	++	+++	+-	++	+-
Staphylococcus warnerii UM224	+-	++	+-	+-	+-	+-	+-	++	+-
Streptococcus anginosus UM241	+-	+-	+-	+-	+-	+-	++	++	++

Note. Initially, the biofilm formation ability was screened, qualitatively, using all 9 growth media presented in this table, including a common media to all isolates - sBHI media, as one of the nutrient-rich laboratory medium. These set of media, was selected, due to being the most common growth media used before in biofilm forming assays. Biofilms obtained were scored from (-) no biofilm formed, (+-) formed medium or good biofilm in all tests, (++) good biofilm formation in all tests, (+++) strong biofilm formation in all tests.
Abbreviations: LB, luria broth; LBG, LB supplemented with 0.25% (w/v) glucose; MRS, de man-rogosa and sharpe agar; MRSG, MRS supplemented with 0.25% (w/v) glucose; TSB, tryptic soy broth; TSBG, TSB supplemented with 0.25% (w/v) glucose; sBHI, BHI supplemented brain heart infusion broth supplemented with 2% (w/w) gelatin, 0.5% (w/w) yeast extract, and 0.1% (w/w) starch; sBHIG, sBHI supplemented with 0.25% (w/v) glucose ; sBHIF, sBHI supplemented with 10% (v/v) fetal bovine serum.

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