Ethanol-Producing Enterocloster bolteae is enriched in Hepatitis B Virus-Associated Gut Dysbiosis: A Case-Control Culturomics Study

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Research Article

Keywords: Dysbiosis, Gut microbiota, HBV virus, Enterocloster bolteae, Culturomics, Metagenomics

Posted Date: June 22nd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3066403/v1
Abstract

Background

Hepatitis B virus (HBV) infection is a global health epidemic that causes fatal complications leading to liver cirrhosis and hepatocellular carcinoma. The link between HBV-related dysbiosis and specific bacterial taxa is still under investigation. Enterocloster is emerging as a new genus (formerly Clostridium), including Enterocloster bolteae, a gut pathogen previously associated with dysbiosis and human diseases such as autism, multiple sclerosis, and inflammatory bowel diseases. Its role in liver diseases, especially HBV infection, is not reported.

Methods

We analyzed the fecal samples of eight patients with HBV and ten healthy individuals using the high-throughput culturomics approach compared to 16S rRNA sequencing. Quantification of ethanol, known for its damaging effect on the liver, produced from bacterial strains enriched in HBV was carried out by gas chromatography-mass spectrometry.

Results

By culturomics, 29,120 isolated colonies were analyzed by MALDI-TOF to identify 340 species (240 species in HBV samples, 254 species in control samples) belonging to 169 genera and six phyla. In the HBV group, 48 species were already known in humans but had not been previously found in the gut, 17 known species not previously found in humans, and six new species were isolated. Comparing bacterial species frequency, we serendipitously found three bacterial genera with significantly enriched bacterial diversity in HBV gut dysbiosis: Enterocloster, Clostridium, and Streptococcus ($p = 0.0016$, $p = 0.041$, $p = 0.053$, respectively). However, metagenomics could not identify this enrichment, possibly concerning its insufficient taxonomical resolution (equivocal assignment of operational taxonomic units). At the species level, significantly enriched species in HBV almost all belonged to class Clostridia as Clostridium perfringens, Clostridium sporogenes, Enterocloster aldenensis, Enterocloster bolteae, Enterocloster clostridioformis, and Clostridium innocuum. Two $E$. bolteae strains, isolated from two different HBV patients, showed high ethanol production (27 and 200 mM).

Conclusion

Culturomics allowed us to identify Enterocloster species, and specifically $E$. bolteae, that are enriched in the gut microbiota of HBV patients. These species have never been isolated in HBV patients so far. Moreover, ethanol production by $E$. Boltea strains isolated from HBV patients could play a role in liver
disease progression. Additionally, culturomics might be critical for better elucidating the relationship between dysbiosis and HBV infection in the future.

**Introduction**

Hepatitis B virus (HBV) infection is a global health burden. Around 257 million people are HBV-positive worldwide [1]. HBV infection constitutes the primary driver of severe complications such as cirrhosis and hepatocellular carcinoma (HCC) [2]. Anti-viral treatment has proven to slow down HBV progression; however, the disease progression occurs in a small percentage of people even when a viral load is undetectable (50 IU/ml) [3]. Therefore, the pathophysiologic mechanisms underlying the evolution of chronic infection with HBV are still incompletely understood and deserve further investigation.

Gut microbiota has a complicated and mutually beneficial relationship with the host and plays a vital role in the host's metabolism, nutrition, pathological processes, and immune function [4]. The link between gut microbiota, its derivatives, and the pathophysiology of the liver has attracted considerable interest [5, 6]. The liver receives various gut-derived substances (bacterial products, environmental toxins, and food antigens) via the biliary tract, portal vein, and systemic circulation due to the structural link to the intestine known as the gut-liver axis [7]. Bacteria and bacterial products from the gut microflora have been associated with systemic inflammation and severe liver diseases [8, 9]. One of those products is ethanol which is known to damage liver sinusoidal endothelial cells and induces centrilobular sinusoidal collapse, which reduces blood flow and impairs the microcirculatory exchange of nutrients and clearance of waste products [10].

In fact, different studies reported significant changes in gut microbiota composition in HBV-infected patients based on metagenomics analysis, characterized by high abundance in *Proteobacteria* phylum [11] and genera such as *Streptococcus* [12], *Prevotella* [13]. *Ruminococcus* and *Veillonella* [14, 15] and low abundance of *Clostridium* [16–18]. However, no study has investigated gut microbiota in HBV-infected patients based on culture methods as culturomics approach. High-throughput culturomics approach has been documented in studying gut microbiota [19]. A previous study has reported the value of culturomics, performed in a case-control study, in defining the missing repertoire of probiotics and beneficial species that could be used in fecal microbiota transplantation (FMT), which requires viable isolated strains [20]. Interestingly, HBV-infected patients who underwent FMT have achieved HBeAg clearance in different studies [21–23]. Additionally, probiotics were reported to function by preventing liver inflammation from impeding the advancement of HCC via antioxidants and anti-metastatic activities [24]. In addition, the role of probiotics, particularly in HBV-related complications such as hepatic encephalopathy, has been established in different studies [25–28]. Therefore, it is essential to understand the distinction between culturomics, which explicitly identifies live organisms and metagenomics which yields DNA sequences most likely originate from already-dead species and cannot be isolated or multiplied [29].
Moreover, among the genus *Clostridium*, a new genus of anaerobic bacteria, namely *Enterocloster*, has emerged as a reclassification due to advances in phylogenetics [30]. *Enterocloster bolteae* (formerly known as *Clostridium bolteae*) is a gastro-intestinal pathogenic bacterium excreting metabolites that are thought to act as neurotoxins [31] and are known to produce ethanol [32]. In patients with HBV infection, alcohol intake is associated with more severe liver disease [33].

In this small study, we are the first to describe a culturomics-based microbiome profile to the species level in HBV-infected patients and healthy individuals. The species identified by both culturomics and metagenomics for each group of samples were compared, highlighting the complementarity of these two approaches. Our study aims to detect possible endogenous ethanol production by specific bacterial strains enriched in HBV-infected patients.

**Material and Methods**

**Study design**

A case-controlled study was carried out in the Hepatology Department of Marseille University Hospital (South-eastern France), Marseille, France, according to STROBE statement guidelines [34]. The study was approved by the local ethics committee of l’Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France (IHUMI, 2020-004), and the approval of the Protection of Persons Committee (Approval No. CPP: 21.04391.000046–21075), and carried out according to the 2013 Declaration of Helsinki (1975; World Medical Association, 2013) [35]. An informed consent was obtained from the study subjects before their enrolment.

**Study population**

The HBV patients were recruited from Marseilles University Hospital. Healthy controls without chronic diseases or on regular medications were recruited using a snowball approach. Data on age, gender, weight, height, and dietary habits were collected. The HBV patients were confirmed to be positive for hepatitis B surface antigen for at least six months; the diagnosis was made according to European Association for the Study of the Liver (EASL) guidelines [36].

Alcoholism and probiotics/or antibiotics intake in the previous month were exclusion criteria for both cases and controls. In addition, vegetarians and lactose lovers were excluded from our study. The other exclusion criteria for all patients were as follows: patients with viral hepatitis other than HBV, solid organ transplantation, smoking, immunosuppressive drugs treatment within six months, acute or chronic infectious diseases, other liver disease, metabolic disease, malignancy, and autoimmune diseases. The fecal samples from all participants were collected and kept in sterile plastic tubes and immediately (within 15 min) stored at -80 °C until used.

**High-Throughput Culturomics Approach**
The stool samples (eight samples from HBV-infected patients and ten samples from controls) were cultured according to the culturomics approach previously established in our laboratory [37]. The methodology of fast culturomics was applied as described by Naud et al. [38]. The modest number of samples used was due to the lengthy time required for each by culturomics analysis (one month). Three culture conditions included two blood-culture bottles supplemented with rumen and sheep blood kept in aerobic and anaerobic conditions and a third bottle supplemented with inhouse YCFA (yeast extract, casein hydrolysate, fatty acids) medium kept in anaerobic condition to mimic the gut environment. The culture bottles were kept incubated on days 1, 3, 7, 10, 15, 21, and 30.

The colonies were identified using Matrix Assisted Laser Desorption/ Ionization Mass Spectrometry (MALDI-TOF/MS) according to the manufacturer's instructions [39, 40]. Each obtained spectrum was matched against the spectra of Bruker's and the Laboratory of La Timone's home database (Accessed on October 2022). A score > 1.9 allowed identification at the species level. In the case of unidentifiable colonies by MALDI-TOF (score < 1.9), DNA from the unidentified colonies was extracted, amplified, purified and analyzed as described in [20]. The sequences with a similarity percentage under 98.65 or 95% [41] were identified as new species or new genera, respectively, and described according to the taxonogenomics concept [42]. If the bacterial species identification was not interpretable, 16S ribosomal DNA gene sequencing was performed.

16S ribosomal DNA gene amplification and sequencing

The samples were treated through two kinds of lysis methods according to a previous study [43]. For each extraction protocol, the 16S "V3-V4" region of DNA samples was amplified by a PCR of 45 cycles, using the Kapa HiFi Hotstart ReadyMix 2x (Roche). The used primers contain a conserved V3_V4 region with overhang Illumina adapters (FwOvAd_341F: 5'TCGTCGGCACGTCAGATGTGTAAGAACGACGCTACGGNGGCWGCAG3'; RevOvAd_785R: 5'GTCTCGTGGGCTCGGAGATGTGTAAGAACGGACTACHVGGGTATCTAATCC3'). Automated cluster generation and paired-end sequencing with dual index reads were performed in a single 39-hour run in a 2x250bp. The paired reads were filtered according to the read qualities. The raw data were configured in fastq files for R1 and R2 reads.

Measurement of Ethanol production by strains enriched in HBV patients

The bacteria were grown in YPD (Yeast Extract-Peptone-Dextrose) broth, but the formula was modified by adding glucose (YPG) instead of dextrose. The proportions of the formula per litre of prepared broth were: 10 g yeast extract (bioMérieux), 20 g peptone (BD Diagnostic) and 20 g glucose. Once all ingredients were thoroughly dissolved, the broth was filtered using the RapidFlow™ filtration system (Thermo Fisher - France) with a 0.22 µm membrane, and 10 mL of filtered broth was poured into Hungate tubes. Each tube was inoculated at a concentration of 1 McFarland to standardize bacterial concentration. Several colonies were picked and suspended in tubes with sterile 0.85% NaCl medium (bioMérieux), and turbidity was measured using a McFarland densitometer.
The strains were grown in 3 different experiments, with some variations in the protocol in terms of inoculated bacterial concentration and degassing time. **Experiment 1**: the bacterial suspension was inoculated at 1 McFarland, and the tubes were degassed for 2 minutes. **Experiment 2**: To promote the growth of *E. bolteae* strains, the concentration of the bacterial suspension was increased to 3 McFarland, the other strains were grown at 1 McFarland, and the degassing time was 2 min for all strains. **Experiment 3**: All strains were grown at 1 McFarland, and the tubes were degassed for 3 minutes. *E. bolteae* strain s28 42 was grown on two different media (YPG and BACT/ALERT® FN Plus flask (bioMérieux, Lyon, France)) with a commercial medium. The BACT/ALERT FN Plus flasks for blood culture contain polymeric beads that adsorb antibiotics with peptones/biological extracts, anticoagulants, vitamins, amino acids, carbon sources, trace elements, and other complex amino acid and carbohydrate substrates in purified water in a vacuum atmosphere of N2 and CO2.

Quantification of ethanol produced in each culture was carried out by headspace gas chromatography-mass spectrometry (HS-GC-MS) using a Turbomatrix HS110 sampler connected to a Clarus chromatograph and a SQ8 single quadrupole mass spectrometer (Perkin Elmer). A standard ethanol curve was prepared with concentrations ranging from 0.5 mM to 100 mM spiked with isopropanol as an internal standard (5 mM). Samples were dispensed into HeadSpace (HS) vials using a 1mL aliquot of each culture, then spiked with isopropanol. HS vials were heated at 60 degrees (10 minutes) and pressurized to 25 psi (1 minute). The volatilized content was transferred to the GC (0.03 minute, split 10/1). Alcohols were then separated through an Elite BAC2 column (30 m, 0.32 ID, Perkin Elmer) maintained at 70 degrees using Helium as carrier gas (19.5 psi). A selected Ion Recording method was used to measure ethanol (31 m/z) and isopropanol (45 m/z). The data obtained were analyzed using Turbomass 6.1 software (Perkin Elmer).

**Bioinformatic analysis**

The raw sequencing data for all samples were deposited into the NCBI Sequence Read Archive database (Accession number: PRJEB62828). Noisy sequencing data were excluded, and chimeric sequences were identified and removed by Chimera Slayer. The clean data were clustered into operational taxonomic units (OTUs) at the 97% similarity threshold using UCLUST algorithm after the removal of singletons. The alpha and beta diversity were calculated using the Microbiome Analyst Platform (https://www.microbiomeanalyst.ca/; accessed on March 2023) [44]. Shared taxa present in all groups were defined as the core microbiota.

**Statistical analysis**

The results are expressed as the mean ± standard deviation. The normal distribution was performed using either D’Agostino-Pearson or the Kolmogorov-Smirnov test, and the homogeneity of the variance was analyzed with the F test. Two-tailed unpaired student’s t-test or Mann-Whitney test were used according to normality. Moreover, Fisher’s exact or Chi-squared test was used to compare proportions. Bilateral Barnard’s exact test was used when the sample size was minimal.
For gut microbiota analysis, differences in the relative abundances of OTUs of dominant bacteria were analyzed using Welch's t-test or Mann–Whitney U test. All statistical significance was accepted at $P < .05$. All analyses were performed with GraphPad Prism Software for Windows (GraphPad Software, San Diego, CA, USA) (version 9.0).

Results

Altered diversity in HBV samples by culturomics

Of the 18 samples, 29,120 colonies were analyzed by MALDI-TOF to identify a total of 340 species belonging to 169 genera, and six phyla (HBV samples ($n = 8$) yielded 240 species and 14,340 colonies ($1,793 \pm 225$ colonies per sample) vs. control samples ($n = 10$) yielded 254 species, and 14,780 colonies ($1,478 \pm 265$ colonies per sample) were isolated; unpaired t-test, $p = 0.0168$). One hundred fifty-four species were shared between HBV and control groups, while 86 species were uniquely detected in the HBV group. A detailed list of the isolated bacterial species is mentioned in (Supplementary File.1).

In HBV samples, five bacterial phyla were isolated, with a majority of Bacillota (148 species), followed by 34 Bacteroidota, 32 Actinobacteria, 16 Proteobacteria, and 2 Synergistetes. In control samples, six phyla were isolated, with a majority of Bacillota (143 species), followed by 50 Bacteroidota, 40 Actinobacteria, 12 Proteobacteria, 1 Synergistetes, and 1 Verrucomicrobia. Additionally, Proteobacteria species were enriched in HBV but not significantly different from the control group. No Verrucomicrobia species were isolated in the HBV group (Table S1). The U/T ratio (the ratio of species found in only one subject on the total number of species in the group) was higher but not significant in the HBV group indicating a high $\beta$-diversity [84/240 (35%) in HBV samples vs. 77/254 (30.31%) in the control group, uncorrected two-tailed chi-squared test, $p = 0.4287$] (Table S1).

The 240 species isolated from HBV samples belonged to 43 different families and 128 different genera. Among them, 9 were the best-represented genera in terms of the diversity in the species, including Bacteroides (11 species), Enterococcus (11), Alistipes (9), Streptococcus (9), Clostridium (8), Peptinophilus (8), Enterocloster (5), and Prevotella (5). In control samples, A total of 111 genera were identified, with the seven most represented genera including Bacteroides (15 species), Enterococcus (11), Alistipes (12), Peptinophilus (9), Bifidobacterium (7), Limosilactobacillus (7), and Clostridium (6) (Table S2). Thirty-four genera have been only represented in the HBV group and not in the control group. We found three bacterial genera belonged to the Bacillota and Bacilli phyla with significantly increased diversity (number of species of this genus per sample) in HBV patients: Enterocloster ($p = 0.0016$), Clostridium ($p = 0.041$) and Streptococcus ($p = 0.053$) (Table S2; Fig. 1). Strikingly, Enterocloster diversity was 11.3 times greater in cases than in controls, whereas it was 3.5 times greater for Streptococcus and 2.3 times greater for Clostridium.

At the species level, the species with the highest significant difference in frequency belonging to class Clostridia were 4 Enterocloster (E. bolteae, E. aldenensis, E. clostridioformis and E. citroniae) and 3
Clostridium (C. perfringens, C. sporogenes and C. innocuum) in HBV group. However, Olsenella uli belongs to Class Coriobacteriia was found to be more frequent in the control group (Fig. 2a). The proportion of positive samples for Enterocloster was higher in the HBV group (7/8 vs. 2/10, \( p = 0.009 \), bilateral Barnard's exact test). Two HBV-infected patients (HBV3, HBV7) were diagnosed with cirrhosis. HBV3 showed a greater diversity of Enterocloster species than the other samples (Fig. 2b).

Overall, we noticed that beta diversity assessed by the U/T ratio was non-significantly high in the HBV group compared to controls; however, it was significantly decreased alongside the aerointolerant and aerotolerant diversity (Table S1). Moreover, the hitherto unknown diversity was assessed and defined as the number of new species added to the species not previously known from the human gut by sample for culturomics analysis and as the number of unidentified OTU for metagenomics analysis [20]. The difference was not significant by culturomics for new species (0.75 ± 1.09 in HBV vs. 1 ± 1.6 in controls, \( p = 0.96 \), as well as for previously known species but which had not been previously found in humans (3.5 ± 1.41 in HBV vs. 2.36 ± 1.07 for controls, \( p = 0.0763 \)) suggesting a similar \( \alpha \)-diversity (Table S3).

Additionally, the species isolated in the HBV group were compared to a recently published cultured human bacteria repertoire describing a total of 3242 bacterial species that have been so far cultured at least once from the human [45]. We found 81 shared species and five species uniquely described in our HBV group. Fourteen species have been found in common between HBV and control groups. Supplementary File.1 demonstrates a complete list of bacterial species all groups share. Moreover, among the 240 species isolated in the HBV group, we found 175 species that are already known in the human gut, 48 species that are already known in humans but had not been previously found in the gut, 17 known species but had not been previously found in humans and six species were identified as new species (Table S4). In the control group, 11 unknown species were isolated from the human gut, among which four new genera were identified (Table S4), 13 are known but had not been previously found in humans, and 44 are already known in humans but had not been previously found in the gut.

Diversity assessed by metagenomics

The metagenomics analysis showed that in the HBV samples 826,517 associated assigned at a prokaryotic species level were distributed into seven phyla (Actinobacteria, Bacteroidetes, Euryarchaeota, Bacillota, Proteobacteria, Saccharibacteria, and Verrucomicrobia). Control samples generated 577,496 associated reads and were divided into the same seven phyla detected in the HBV group. These reads matched 682 species in the HBV group and 715 in the control group. Alpha diversity was not significantly increased in the HBV group by calculating Chao1 (\( p = 0.168 \)) and Shannon indexes (\( p = 0.315 \)). No significant difference between the two groups except for Proteobacteria, which were significantly decreased in the HBV group, \( p = 0.0342 \) (Fig. 3). The HBV group showed 76 genera while the control group showed 74 genera (g_IHU_PG_93_Eubacteriaceae_207 and g_Atribacter3 were absent from the control group) (Table S5). Highly significant different genera in both groups with LDA score 2.0 and \( P < 0.05 \) have been illustrated in Fig. 3. Interestingly, no significant difference was reported regarding abundance for the same frequent genera detected by culturomics as Clostridium (\( p = 0.145 \)) and Streptococcus (\( p =
Enterocloster was not recognized as an abundant taxon by Linear discriminant analysis (LDA). Highly abundant OTUs assigned to the species level with LDA score 2.0 and p < 0.05 in both groups are shown in Fig. 3.

Notably, 6 out of all seven species belonging to Enterocloster and Clostridium genera could be identified as multi-assigned OTUs in the dataset of 16S ribosomal RNA sequencing results (Enterocloster_aldenensis, Enterocloster_bolteae, Enterocloster_citroniae, Enterocloster_clostridioformis). Enterocloster_bolteae couldn’t be identified as a single OTU. Clostridium_innocuum was identified as a single OTU, while Clostridium_perfringens was identified in single and multi-assigned OTUs. C. sporogenes was not identified at all in the samples. Clostridium_innocuum and Clostridium_perfringens species were unequivocally identified (only one species known for this OTU). Three species were identified equivocally (Fig. 4). Overall, none of the seven species with significant differences identified by culturomics had a considerable difference by sequencing. Similarly, no significant differences were identified when we evaluated the relative abundance of all OTUs attributed to Enterocloster or Clostridium at the genus level.

The metagenomics analysis showed a decreased aerotolerant α-diversity (1.11 ± 0.40 in HBV vs. 1.21 ± 0.49 in controls; p = 0.514) in addition to a decreased anerointolerant diversity in the HBV group (2.85 ± 0.53 in HBV vs. 3.08 ± 0.31 in controls; p = 0.359). These results confirmed the specific decrease in anaerobic diversity found by culturomics, indicating loss of anaerobic species in patients with HBV infection. The hitherto unknown diversity assessed was consistently and significantly increased in the HBV group as unidentified OTUs were higher in HBV (p = 0.0434). Additionally, at the prokaryotic level, 18.65% of all reads in the HBV group were not assigned vs. 25.67% in control (p < 0.0001).

**Missing Repertoire in HBV-infected Patients**

For identification of potential probiotic species, we considered all of the bacterial species that were identified both by culturomics and metagenomics in the control samples but not in HBV samples (Supplementary File.1). The common species between both approaches in the control group were six including Alistipes merdae, Christensenella massiliensis, Dialister succinatiphilus, Fenollaria timonensis, Mediterranea massiliensis, and Metaprevotella massiliensis. However, the HBV group showed nine shared species between both approaches including Clostridium marseillense, Mogibacterium neglectum, Mogibacterium vescum, Pantoea agglomerans, Prevotella caccae, Prevotella copri, Terrisporobacter glycolicus, Weissella cibaria, and Weissella confusa.

Comparing the species identified by metagenomics and culturomics in HBV and control groups, 213 species were found exclusively in the control samples. (Supplementary File.1). These species belonged overwhelmingly to the Bacillota phylum (89; 41.75%) and Proteobacteria (73; 34.27%), followed by a low number of species from the Actinobacteria (22; 10.33%), the Bacteroidetes (26; 12.21%), and Euryarchaeota (1; 0.47%) phyla. Among the missing repertoire, strikingly, 129 species (60.56%) were strictly anaerobic.
We searched through the literature for each of these species to find a possible probiotic use for humans. Three species, *Limosilactobacillus oris*, *Propionibacterium freudenreichii*, and *Streptococcus oralis* were found to have possible probiotic features (Table S7). Probiotic features included short-chain fatty acid production, antioxidant metabolism, and antibacterial potential. Two of them (*L. oris* and *S. oralis*) were isolated by culturomics in our control samples and are readily available in our laboratory’s Collection de Souches de l’Unité des Rickettsies (CSUR) collection.

**Ethanol quantification produced by Enterocloster species**

In culturomics, at the strain level, we studied the ethanol production of HBV patient strains (strain s2032 from patient HBV4 and strain s2842 from patient HBV6). Following two attempts in YPG and BACT/ALERT FN media, the third effort using a closed BACT/ALERT FN bottle to establish controlled anaerobiosis with an optimized medium (a technique developed during microbial culturomics) [46], showed significant ethanol production (Fig. 5). No significant growth was observed for any of the *E. bolteae* strains or *E. citroniae* in experiment 1 and 2 (Table S8). However, in experiment 3, *E. aldenensis* strain s16 38 and *E. bolteae* strain s28 42, exhibited weak microbial growth on YPG medium. Interestingly, *E. bolteae* strain s28 42 showed significantly higher ethanol production when growing on FN medium (Table S8).

**Discussion**

In this study, we report four HBV-enriched *Enterocloster* species using culturomics approach for the first time in the characterization of gut microbiota in HBV-infected patients rather than metagenomics. To our knowledge, no *Enterocloster* species have been linked to HBV infection.

The “culturomics” approach, whose effectiveness in analyzing gut microbiota has not yet been established [19], offers a significant benefit over metagenomics to exclude the enormous number of ingested bacteria that are killed in the upper gut by the acidic environment and bile salts [47] and to provide live strains on which further analysis can be performed. The popular method for examining the diversity of gut microbiota is metagenomics; however, these studies' results have very low reproducibility, likely due to variations in sampling, DNA extraction methodology, sequencing method, and data analysis techniques [48]. Regardless of the discrepancy between metagenomics and culturomics [49, 50], culturomics allowed the extension of gut microbiota known diversity and functions [51]. Furthermore, culturomics is a validated technique with consistent species identification that avoids one of the previously mentioned disadvantages of the metagenomic approach, particularly poor taxonomic resolution [52].

We identified a total of 240 and 254 living, viable, and cultivable bacterial species in the HBV and control groups, respectively. Fifty-five species have been recognized as core microbiome detected by both approaches. Among them, 213 species formed a missing repertoire in HBV-infected patients since they were found by both techniques exclusively in the healthy control group but not in the HBV group. The global beta diversity, by culturomics, was significantly decreased alongside the aerointolerant diversity in
the HBV group compared to controls. Metagenomic results showed the same diversity pattern. Regarding the hitherto unknown diversity, it suggested a similar α-diversity by cultuomics, yet it was significantly increased by metagenomics in HBV group. Nevertheless, some studies using the metagenomic approach showed different results. Joo et al. reported higher alpha diversity in the HBV group [53], while Zheng et al. reported its decrease [54]. This could be attributed to differences in sample sizes and study populations.

Gut microbiota at the phylum level showed a non-significant increase in Proteobacteria and a decrease in Actinobacteria and Bacteroidetes in the HBV group by culturomics. However, metagenomics showed a significant reduction in Proteobacteria and a non-significant increase in Bacteroidetes. Indeed, several studies support our culturomics findings, which show a continuous increase in the abundance of Proteobacteria in HBV-infected patients [16, 55]. According to other research, Bacteroidetes and Actinobacteria in the HBV group either increased or decreased [15, 16, 55]. Interestingly, both approaches reported an increase in Bacillota, which was in agreement with the previous studies [14, 54]. Therefore, further exploration of gut microbiota characteristics in extensive HBV-related studies is warranted.

Among the top highly represented genera by culturomics, Enterocloster and Clostridium were significantly increased in the HBV group. However, metagenomics showed a significant abundance of different genera, including Roseburia, Kandleria, and Atribacter, in the HBV group. In fact, our results are different from most previous studies, which reported a decrease in Clostridium [11, 56, 57] and Roseburia [15, 57] genera in HBV-infected patients. This could be attributed to the different sample size and diagnostic approaches we used in our study. Kandleria, a genus from the family of Erysipelotrichidae [58], and Atribacter, a genus of the candidate phylum Atribacterota [59], have not been reported before in patients with liver disease as HBV infection. In agreement with many previous studies [14, 15, 57, 60], Streptococcus was increased in our HBV group by culturomics, although non-significantly.

Enterocloster is a genus recently identified in 2019 [30] as a reclassification of Clostridium genus, thanks to improved genomics and taxonomy. This new genus was recently identified by an unbiased study as the main supplier of inoviruses [61], which are potentially pathogenic prophage viruses [62, 63]. This genus comprises six validated species [30], including E. aldenensis, E. asparagiformis, E. bolteae, E. citroniae, E. clostridioformis and E. lavalensis. We found five species in HBV samples and only 2 in controls. Four species (E. aldenensis, E. bolteae, E. citroniae and E. clostridioformis) had a significantly increased frequency in the HBV-infected group. Notably, both E. clostridioformis/E. bolteae and E. asparagiformis/E. lavalensis were closely related based on phylogenetic, phylogenomic, and phenotypic perspectives [30]. Culturomics alone could separately identify those species, not distinguishable by sequencing. A total of 18 OTUs have been identified as Enterocloster species, and 448 OTUs were assigned for Clostridium species. Among 18 OTUs of Enterocloster species, 7 OTUs were multi-assigned for E. asparagiformis and E. lavalensis. E. bolteae has been identified in 3 multi-assigned OTUs.

Species of Enterocloster genus have been associated with different diseases and dysbiosis. Enterocloster aldenensis was reported in intra-abdominal infections [64]. Additionally, a high carbohydrate fermenting
Enterocloster species such as *E. clostridioformis* was documented to be associated with clinical bacteremia cases [65] and was highly abundant in Type II diabetes [66] and Crohn's disease [67]. Interestingly, *E. bolteae* was reported in different neurological diseases such as autism [68, 69], multiple sclerosis [70], and neuromyelitis optica spectrum disorders [71]. According to a prior study, *E. bolteae* could produce microbially conjugated bile acids that contribute to the severity of Crohn's disease and irritable bowel syndrome (IBS) [72]. Additionally, *E. bolteae* has been identified as a mediator in fatty acids (FA) acylation to isoBAs (bile acids) [73]. *E. bolteae* has not been reported in HBV infection, and the role of FA-isoBAs in host physiology, their contribution to gastrointestinal or hepatic diseases is under investigation. Surprisingly, among *Enterocloster* species, *E. citroniae* was described as enriched purine-degrading species and considered a promising therapeutic prebiotic to reduce serum uric acids levels in a clinical trial performed on renal failure patients [74]. Moreover, patients on peritoneal dialysis who experienced a restriction of advanced glycation end products diet (in order to decrease cardiovascular disease incidence) showed an increase in *E. citroniae*, suggesting its beneficial role [75].

Our culturomics results also showed a high significant abundance in three *Clostrium* species, including *C. perfringens*, *C. innocuum* and *C. sporogenes* in the HBV group. In fact the genus *Clostridium* has recently been taxonomically clarified [76], making it possible to specify the associations between this genus and several diseases. In particular, *C. perfringens* is associated with necrotizing enteritis, enterotoxemia, and gas gangrene [77]. Moreover, cirrhotic patients infected with *C. perfringens* manifested poor prognosis[78]. *Clostridium innocuum* was described as an extraintestinal pathogen causing bacteremia, endocarditis, osteomyelitis, and peritonitis and may also cause a *C. difficile*-like antibiotic-associated diarrheal illness [79]. Moreover, *C. sporogenes* was able to transform tryptophan into indole-3-propionic acid affecting intestinal permeability, and was found to be negatively correlated with several metabolic diseases [80]. Therefore, the potential role of those particular species deserves further investigation in HBV-infected patients.

Additionally, our study reported different highly abundant species in the HBV group by metagenomic such as *Coprococcus_eutactus* (OTU731), *Parabacteroides_distasonis* (OTU38338), *Ruminococcus_torques* (OTU38812), *Kandleria_vitulina* (OTU34133) and multiassigned OTUs for *Streptocoocus* (OTU36481) and *Bifidobacterium* (OTU37693) species. Surprisingly, both culturomics and metagenomics approaches identified nine and six species in HBV and control samples, respectively. However, those species were not significantly frequent or abundant except for *P. copri* (OTU38502), which was found to be highly abundant in the HBV group by metagenomics. Different studies have reported an increase in *Prevotella* genus in HBV-infected patients [12, 15]. *Prevotella* was also reported to be related explicitly to the immunotolerant phase of HBV infection [81], indicating that members of *Prevotella* genus could play a vital role in viral escape from the host immune system.

Under anaerobic conditions, it has been reported that bacteria belonging to the genera *Escherichia*, *Bacteroides*, *Bifidobacterium*, and *Clostridium* can produce ethanol by fermentation from consumed carbohydrates [82]. Moreover, *E. bolteae* species was particularly interesting because it has been reported that bacteria from the *Clostridia* class carry genes encoding for ethanol production pathways [83].
Interestingly, *E. bolteae* has been described as an opportunistic pathogen in humans [82] and in situations of dysbiosis, such as those arising in diseases like NAFLD (non-alcoholic fatty liver disease), it promotes liver damage through endogenous ethanol production [84] via increasing the permeability of the gut epithelial barrier. Consequently, *E. bolteae* strains were re-cultured, but the inoculum concentration increased to 3 MacFarland (experiment 2) to promote growth. However, no ethanol was detected in any *E. bolteae* strain, but slight bacterial growth was observed due to the turbidity of the medium. We speculated that this might be a problem linked to the anaerobic environment of the tube, and we decided to increase the degassing time to 3 min. The results of experiment 3 showed ethanol production in all the strains. Surprisingly, *E. bolteae* strain s28 42, grown on FN commercial medium, produced the highest ethanol. We assume the previous deficit in *E. bolteae* growth could be attributed to the amount of liquid medium used (40 mL in the commercial medium vs. 10 mL in YPG medium). We suggest that the ethanol production in FN medium is due to its higher nutritional content than YPG medium.

In conclusion, characteristics of the HBV-associated dysbiosis in our study showed an increase in alpha diversity, a depletion of aerointolerant diversity, and enrichment in potentially pathogenic *Enterocloster* and *Clostridium* species. In this context, the present results suggest that endogenous alcohol production by gut microbiota might participate in HBV-related liver disease, as recently described in non alcoholic steato-hepatitis patients [85]. Additionally, microbial culturomics allows us to obtain live bacterial species that could contribute to the pathophysiology of the disease.

Future studies should also focus on elucidating the mechanisms by which *E. bolteae* might contribute to liver inflammation and HBV disease progression and exploring interventions to restore healthy gut microbiota in HBV-infected individuals.

Finally, our results open new insight into microbiota's potential role in the pathophysiology of HBV-related disease, paving the way for further research regarding microbiome-targeted therapeutic options such as probiotics and FMT in HBV-related liver fibrosis, cirrhosis, or cancer.

### Declarations

#### Funding

This work was funded by ANR-15-CE36-0004-01 and by ANR “Investissements d’avenir”, Méditerranée Infection 10-IAHU-03 and was also supported by the Région Provence-Alpes-Côte d’Azur. This work received financial support from the Fondation Méditerranée Infection.

#### Data Availability Statement

The metagenomic files are available online under bio project number PRJEB62828. All the cultured species in this study are preserved and available for further investigation. They are deposited in the Collection de Souches de l’Unité des Rickettsiesand (CSUR) collection numbers as *Enterocloster bolteae* Q5636, *Clostridium sporogenes* Q544, *Clostridium sporogenes* Q5652.
Acknowledgments

Our thanks go to all the staff of the hepatology unit.

Conflicts of Interest

The authors would like to declare that Didier Raoult has been a consultant in microbiology for the Hitachi High-Tech Corporation from March 2018 until March 2020. Otherwise, the authors declare no other conflict of interest.

Author contributions

R.M.W. contributed to the investigation, formal analysis, original draft writing, and visualization.

B.M. performed culturomics of the control samples.

P.B. contributed to the study design, review, and editing.

M.T.A. contributed to the review & editing.

M.M.R. performed ethanol measurement.

A.C. contributed to bioinformatic analysis.

C.A. contributed to 16s RNA sequencing.

N.A. contributed to the formal analysis of ethanol.

D.R. contributed to the conceptualization and project administration.

M.M. contributed to conceptualization, supervision, formal analysis, writing, review & editing, project administration, and visualization.

R.G. contributed to the study design, review, and editing.

All authors have read and agreed to the published version of the manuscript.

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**Figures**
Figure 1

Boxplot showing the number of species per sample identified by culturomics in the three genera with a significant frequency difference between hepatitis B virus (HBV) infected group (Red) versus the control group (Green). Two-tailed Mann-Whitney test; * $p$ value < 0.05. ns: not statistically significant.

$\triangle$ HBV (n = 8)
$\triangledown$ CONTROLS (n = 10)
**Figure 2**

**a)** Species with a significant frequency difference between hepatitis B virus (HBV) and control samples by the culturomics approach. **b)** *Enterocloster* increased diversity in the HBV group. **c)** Detailed *Enterocloster* species isolated per sample. **d)** Proportion of samples positive for *Enterocloster* species. Barnard's bilateral exact test was used to test the \( p \)-value. * \( p \)-value <0.01.

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**Table:**

<table>
<thead>
<tr>
<th>Species</th>
<th>Enterocloster</th>
<th>Frequency difference</th>
<th>HBV (n=8)</th>
<th>CTL (n=10)</th>
<th>p-value</th>
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<td>Enterocloster baltense</td>
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<td>Enterocloster clostridiformis</td>
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<td>Clostridium inacuum</td>
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<td>Enterocloster citroniae</td>
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**Diagram a:**

- **HBV3**
- **HBV7**

**Diagram b:**

- **HBV (n=8)**
- **CTL (n=10)**

- Barnard bilateral exact test
  - \( P = 0.009 \)

**Diagram c:**

- **HBV3 & HBV7 = Cirrhosis**

**Diagram d:**

- **HBV (n=8)**
- **CTL (n=10)**
Figure 3

Relative abundance of gut microbiota in the fecal samples of the HBV-infected group (n=8) and the control group (n=10). (A) barplot expressing abundant phyla. (B) genus level and (c) species level were selected via LEfSe (LDA score > 2). Some OTUs correspond to several species (see Table S6). For clarity, only one clinically relevant species is represented on the graphic. **HBV**: Hepatitis B virus; **LEfSe**: linear discriminant analysis effect size.
discriminant analysis effect size; **LDA**: linear discriminant analysis; OTUs: operational taxonomic units. *P < 0.05.

**Figure 4**

*Enterocloster* and *Clostridium* species detected in 16s RNA sequencing data file per sample. **OTUs**: Operational Taxonomic Units; **HBV**: Hepatitis B virus- infected patients; **CTL**: control. *E. bolteae, E. citroniae* and *E. clostridioformis* were identified equivocally.
Figure 5
Detection of ethanol by gas chromatography-mass spectrometry (GC-MS) produced by isolated Enterocloster strain S2842 in HBV using YPG or BACT/ALERT® FN Plus culture media. The first chromatogram shows the analysis of *E. bolteae* strain s28 42 grown on YPG medium (10% glucose), in which bacterial growth was deficient and no ethanol was detected. The second chromatogram corresponds to the same strain grown on a commercial medium (BACT/ALERT® FN Plus), in which
bacterial growth was evident within 24h, and the presence of ethanol was observed at the peak at 5.09. The peak at 5.58 in both graphs corresponds to the isopropanol used as an internal standard.

**Supplementary Files**

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- SupplementaryFile1.xlsx
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- TableS2.xlsx
- TableS3.docx
- TableS4.docx
- TableS5.xlsx
- TableS6.xlsx
- TableS7.docx
- TableS8.docx