Gut Pathogens

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Thomasclavelia ramosa and alcohol-related hepatocellular carcinoma: a microbial culturomics study

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Abstract

Background Gut microbiota alteration is implicated in the pathogenesis of alcoholic liver disease (ALD) and associated hepatocellular carcinoma (HCC). No study has characterized the dysbiosis associated with ALD by microbial culturomics, which certifies viability and allows pathobiont strain candidates to be characterized.

Methods A case-control study ($n=59$) was conducted on patients with ALD without HCC (ALD-NoHCC, $n=16$), ALD with HCC (ALD-HCC, $n=19$) and controls ($n=24$) groups. 16 S rRNA amplicon sequencing and microbial culturomics were used as complementary methods for gut microbiome profiling.

Results Compared to the control group, *Thomasclavelia ramosa* and *Gemmiger formicilis* were significantly increased in the ALD-HCC group and *Mediterraneibacter gnavus* was significantly increased in the ALD-NoHCC group using 16 S rRNA sequencing. By microbial culturomics, *T. ramosa* was detected in all ALD samples (100%), and the most enriched since cultivated in only a small proportion of controls (20%, $p<0.001$).

Conclusions *T. ramosa*, identified by culturomics and 16 rRNA sequencing, may be associated with ALD and ALD-HCC. These results highlight the potential role of *T. ramosa* in liver cancer, in line with its genotoxic properties and its tumor growth-promoting effect in gnotobiotic mice recently reported.

Highlights

- The gut microbiota signature of ALD and ALD-HCC was explored by microbial culturomics and 16 S amplicon sequencing.
- By culturomics, *T. ramosa* was the most enriched and cultured from all included ALD patients, but in only 20% of controls ($p<0.05$).
- By 16 S amplicon sequencing and linear discriminant analysis, *T. ramosa* was the most enriched in ALD-HCC patients.

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- *T. ramosa* has recently been reported to have genotoxic properties in vitro and to promote the development of colon tumors in vivo.
- *T. ramosa* is identified as a putative oncobiont associated with ALD-HCC, thus opening new avenues for diagnosis and treatment.

Keywords *Thomasclavelia ramosa*, *Enterocloster bolteae*, *Mediterraneibacter gnavus*, Alcohol-related liver disease, Hepatocellular carcinoma, Gut microbiome, Microbial culturomics, 16S rRNA sequencing, Cancer

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer (90% of cases), primarily associated with chronic liver disease. Its major risk factors include chronic viral hepatitis infection (HBV, HCV), Metabolic dysfunction-associated Steato-Hepatitis (MASH) and Alcoholic Liver Disease (ALD) [1]. Liver cancer ranks sixth for incidence and third for mortality across diverse cancers, which has caused a great cancer burden globally [2].

The link between alcohol, liver disease, and cancer is well-established. Its mechanism would include direct toxicity of alcohol on the liver, but persistent instrumental factors are suspected, as the evolution of the disease is not reversible upon withdrawal [3]. The gut-liver axis and gut microbiota are potential candidates to explain the persistence of a vicious circle that would explain the persistent excess risk up to 10 years after weaning [4]. Recent studies suggest that alcohol dependence syndrome and ALD are both associated with gut microbiota alteration with distinct features [5, 6]. This indicates that a specific and persistent gut dysbiosis may enhance cirrhosis and hepatocarcinogenesis by the gut-liver axis. Indeed, some data from human studies [7–12] and animal experimental models [13–18] indicate that HCC occurrence is related to gut microbiota, and treatment with broad-spectrum antibiotics decreases HCC tumor growth in mice [19].

Several metagenomic studies have investigated gut dysbiosis in patients with ALD based on 16 S rRNA amplicon sequencing [20–22] or shotgun (whole-genome) sequencing [6]. However, no study described gut dysbiosis in patients with ALD or ALD-associated HCC (ALD-HCC) based on the culturomics approach. It has been shown that the reliability of metagenome assembled genomes (MAGs) obtained through deep sequencing is limited and biased by the production of erroneous MAGs compared to isolation especially for the handful of MAGs that are critical for the study of pathogens [23]. This evidences that culture remains essential and complementary to sequencing when investigating gut pathogens associated with liver cancer.

Culture-based studies focusing on intestinal dysbiosis and liver cancer are scarce. We found a study reporting cultured microbial counts but with minimal microbial taxonomic accuracy and evidencing an *Escherichia coli* enrichment [7]. Microbial culturomics is a new -omics strategy developed in our center as a high-throughput culture method based on Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and diversified physicochemical culture conditions mimicking the natural microenvironment [24]. Our team applied fungal and bacterial culturomics in the context of liver diseases, demonstrating the importance of ethanol-producing yeast and bacteria in MASH and HBV-associated liver disease [25, 26].

However, to our knowledge, no study has yet characterized microbiota associated with ALD using microbial culturomics. We, therefore, decided to carry out this microbial culturomics study to complete the spectrum of dysbiosis of the gut microbiota, whose instrumental role had been demonstrated in ALD with experimental evidence [27]. Accordingly, this study aims to characterize the microbial signature in patients with ALD and ALD-associated HCC using both culturomics and large-scale sequencing (v3v4 region 16 S rRNA amplicon sequencing).

Methods

Full methods are reported in the supplementary data (Supplementary Material 1). Briefly, 59 participants were investigated, including 35 patients with ALD (19 with HCC) and 24 controls (CTL). Liver stiffness measurements in cirrhotic patients were conducted using a FibroScan® instrument (Echosens, Paris, France). Routine biochemistry, including prothrombin index, platelets count, total bilirubin, serum albumin, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, alkaline phosphatase and serum creatinine, were measured. These analyses were performed only among cases. The fecal samples from all participants were collected and analyzed by v3-v4 16 S rRNA amplicon sequencing. We selected 11 samples from patients with ALD and 10 samples from controls for microbial culturomics. For culturomics, 21 samples were selected as this approach is of unequaled value since it is the only one that ascertains viable strains, but it is associated with a huge workload (6 weeks by sample). Statistical analyses were performed using GraphPad Prism Software for Windows (GraphPad Software, San Diego, CA, USA) (version 9.0). P values less than 0.05 were considered significant differences.

Main results

The bacterial genera *Thomasclavelia*, *Enterocloster*, *Clostridium*, and *Peptoniphilus* are associated with ALD by culturomics

The characteristics of the study participants and the general results of culturomics are detailed in the supplementary results. Overall, twenty-one samples were analyzed using microbial culturomics (5 ALD-HCC, 6 ALD-NoHCC, and 10 CTL), allowing the isolation and identification of 32,088 colonies (Table S1). Diversity at the species level was found to be increased in the ALD group for only four bacterial genera, all belonging to one phylum (*Bacillota*), including *Thomasclavelia* ($p = 0.0016$), *Enterocloster* ($p = 0.0058$), *Clostridium* ($p = 0.0021$) and *Peptoniphilus* ($p = 0.0044$, Fig. 1a).

Thomasclavelia ramosa is the most enriched species in ALD by culturomics

At the cultured species level, surprisingly, *T. ramosa* was detected in all ALD patients (11/11 (100%), including those with ALD-HCC ($n = 5$) and ALD-NoHCC ($n = 6$)), but in only 20% of the controls (2/10, two-tailed Fisher exact test $p = 0.00044$, Fig. 1b). Moreover, among all the cultured species, *T. ramosa* also had the most significant difference in frequency between cases and controls (100% vs. 20%: +80%). This was consistent with the increased diversity of the *Thomasclavelia* genus, as reported above (Fig. 1a).

Enrichment of *Enterocloster* species in ALD by culturomics

We recently reported an enrichment in *Enterocloster bolteae* in liver diseases associated with metabolic-associated steatohepatitis [26] and hepatitis B virus [28]. Here, we found again an enrichment in *Enterocloster bolteae* in liver disease associated with alcoholism (Fig. 1b). This was consistent with the increased diversity of this genus in ALD (Fig. 1a), particularly the significant increase of 3 species of this genus, including also *E. clostridioformis* and *E. aldenensis* (Fig. 1b and c). Strikingly, this association was found only by culturomics but not by 16 S rRNA sequencing, as observed in our previous studies [26, 28].

The species *Thomasclavelia ramosa* and *Mediterraneibacter gnavus* are enriched in ALD both by culturomics and 16 S rRNA amplicon sequencing

Linear discriminant analysis (LDA) effect size modeling was applied on the v3v4 sequencing data of the 59 samples (Table S2) to identify specific bacterial taxa associated with ALD. Compared to the control group, the gut microbiota of all ALD patients showed a significantly increased abundance of *Streptococcus_salivarius/Atribacter_sp223* (OTU39314), *Escherichia_albertii / Escherichia_coli* (OTU2689), *Mediterraneibacter_gnavus* (formerly, *Ruminococcus_gnavus* [29]), and *Thomasclavelia_ramosa* (formerly *Clostridium ramosum* [30]). Accordingly, the only two species enriched in ALD both by culturomics and 16 S rRNA sequencing were *T. ramosa* and *Mediterraneibacter gnavus*.

Thomasclavelia ramosa was the only species identified by culturomics and sequencing and associated with hepatocellular carcinoma

Including all three groups (ALD-NoHCC, ALD-HCC, CTL) in a Linear Discriminant Analysis, Mediterraneibacter_gnavus [31, 32] was significantly increased in ALD-NoHCC, while Thomasclavelia_ramosa and Gemmiger_formicilis were the only 2 operational taxonomic units (OTUs) significantly associated with the ALD-HCC group (Fig. 2). Accordingly, *T. ramosa* was the only species identified by culturomics and sequencing and associated with ALD-HCC, identifying it as one of the best oncobiont candidates for further exploration of an instrumental role of a gut microbe for liver cancer associated with alcoholism. Genomic analysis did not identify a clonal specificity (Supplementary Tables 2 & Supplementary Fig. 2).

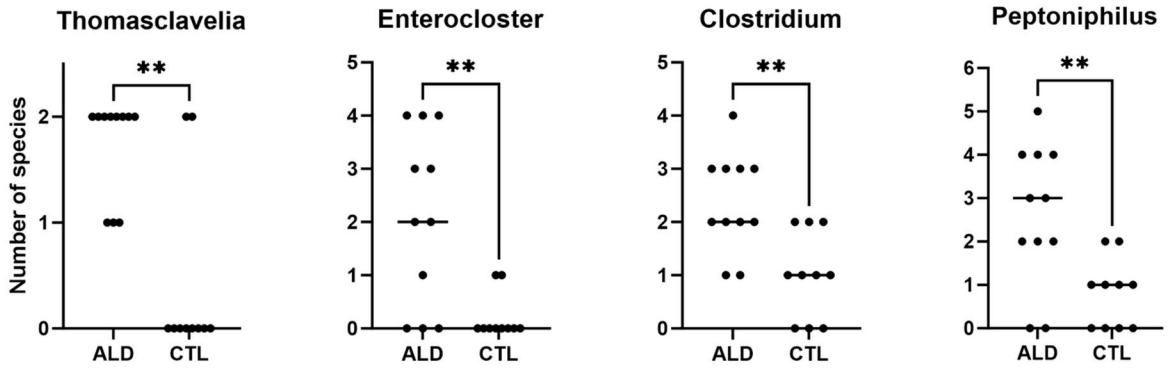
Discussion

This brief preliminary report, which uses a combined approach of microbial culturomics and 16 S rRNA sequencing, identifies *T. ramosa* as one of the best candidates for the intestinal pathogenic oncobiont associated with ALD and HCC associated with ALD. *T. ramosa* is a human gut pathogen associated with several cases of severe infection (bacteremia, infection of aortic aneurysm, osteomyelitis, arthritis, gas gangrene, Fournier's gangrene, fatal infections) recently associated with human cancer, notably colorectal cancer [33, 34], but also HCC in a 2023 Chinese study [35].

One of the first discovered genotoxin produced by a gut commensal was colibactin from *Escherichia coli* strains which alkylates DNA, and associated with colon cancer [36]. A another recent study reported that two other gut commensals, *Morganella morganii* and *T. ramosa*, exhibited genotoxicity and promoted tumor growth in an experimental model [37]. While indolimines have been characterized as the genotoxic molecules for *M. morganii*, the small molecules (< 3 kDa) that lead to the genotoxic and pro-cancer role are not known for *T. ramosa* [37]. Notably, *T. ramosa* lacks known biosynthetic gene clusters (BGCs) and induces DNA damage via colibactin-independent and indolimines-independent mechanisms [37].

In this study, among the 2 species associated with HCC by sequencing (59 samples), only *T. ramosa* was identified in culture (13/21 samples, 11/11 in cancer vs. 2/10 in controls). *Gemmiger formicilis* was not isolated in any sample. Even if we did not succeed in cultivating it here,

a.



b.

Species isolated by culturomics	Frequency Difference	ALD (n=11)	Control (n=10)	p-value
Thomasclavelia ramosa		11	2	***
Enterocloster bolteae		7	0	**
Enterocloster clostridioformis		6	0	**
Oscillibacter massiliensis		7	1	*
Thomasclavelia [Clostridium] innocuum		8	2	*
Hungatella hathewayi		8	2	*
Peptoniphilus harei		5	0	*
Mediterraneibacter gnavus		5	0	*
Clostridium perfringens		6	1	*
Finegoldia magna		6	1	*
Limosilactobacillus fermentum		7	2	
Anaerococcus vaginalis		4	0	*
Anaerostipes caccae		4	0	*
Clostridium sporogenes		4	0	*
Enterocloster aldenensis		4	0	*
Facklamia hominis		4	0	*
Peptoniphilus grossensis		4	0	*
Peptoniphilus meridionalis		4	0	*
Streptococcus constellatus		4	0	*
Phascolarctobacterium faecium		0	4	*
Alistipes communis		3	8	*

Genera with a significant diversity difference between ALD and CTL
Thomasclavelia
Enterocloster
Clostridium
Peptoniphilus

c.

Genus diversity per sample	ALD-NoHCC						ALD-HCC					Control									
	OH1	OH2	OH4	OH6	OH7	OH21	OH3	OH5	OH8	OH9	OH10	CTL13	CTL14	CTL7	CTL8	CTL15	CTL17	CTL4	CTL6	CTL12	CTL21
Thomasclavelia	2	2	2	2	2	1	2	2	2	1	1	0	0	0	0	0	0	2	0	2	0
Enterocloster	4	4	2	1	3	0	4	3	0	0	2	0	1	0	0	0	0	0	0	1	0
Clostridium	4	3	3	2	3	2	1	3	2	2	1	1	1	1	2	0	1	0	2	2	0
Peptoniphilus	5	3	3	4	2	4	2	4	0	0	2	1	0	0	2	0	1	0	1	2	1

Fig. 1 Culturomics results evidenced *Thomasclavelia ramosa* as the gut bacteria the most enriched in ALD. (**a**) Species diversity by genus (only four genera with a significant difference are shown). (**b**) Species with a significantly different frequency of detection between ALD and controls. Barnard's bilateral exact test $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. (**c**) Number of species for each of the four genera associated with ALD for each participant

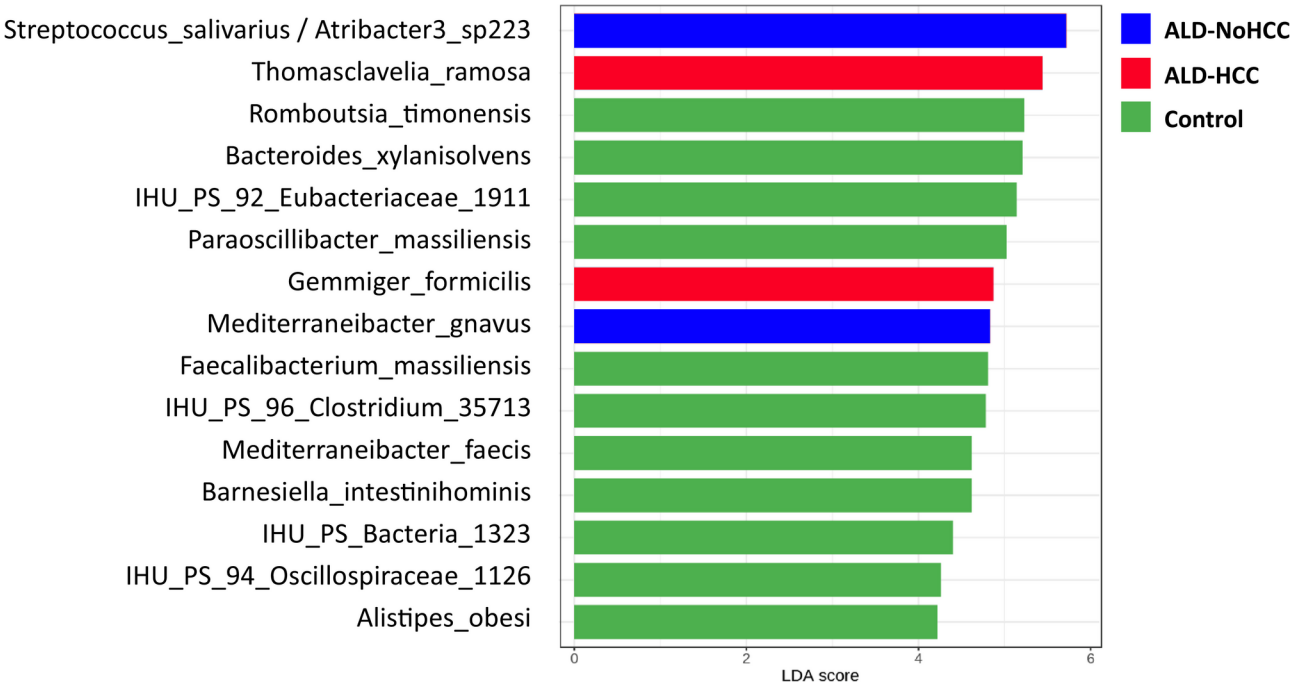


Fig. 2 Linear discriminant analysis (LDA) on 16 S rRNA sequencing results identified association between *Thomasclavelia ramosa* and liver cancer in alcohol liver disease patients. ALD: Alcoholic liver disease; CTL: Controls; HCC: Hepatocellular carcinoma. A logarithmic LDA score > 2 indicated a higher relative abundance in the corresponding group than in other groups

it cannot be ruled out that *G. formicilis* is also associated with liver cancer. Indeed, *G. formicilis* has been associated with colon cancer [38]. Moreover, in a metagenomic meta-analysis on inflammatory bowel disease (IBD), 3 species were associated with IBD: *Asaccharobacter celatus*, *Gemmiger formicilis*, and *Erysipelatoclostridium ramosum* (former name of *T. ramosa*) [39]. This association of *G. formicilis* and *T. ramosa* enriched in a disease associated with an excess risk of cancer (IBD and colon cancer) is consistent with our results.

Limitations of our study included a small sample size and lack of experimental evidence. However, the clear *T. ramosa* signature (LDA score > 5, $p < 0.05$) identified here with a limited sample size means that the difference (effect size) is huge, supporting the strength of the association as the first Bradford-Hill criteria for causality. Consistency and reproducibility are fulfilled by two previous studies confirming the association with CRC [33, 34] and the confirmation of the *T. ramosa*-liver cancer association by another team [35]. The linear discriminant analysis fulfills the biological gradient (the higher *the T. ramosa* 16 S rRNA number of reads - the higher the risk of HCC). The plausibility and experimental evidence are supported by the recently reported in vitro genotoxicity and in vivo tumor growth-promoting properties of *T. ramosa* [37].

The robustness of our results is not due to the number of samples or deep sequencing, but to microbial culturomics, the only approach to certify the presence of viable and biologically active microbes with high fidelity taxonomic characterization at the species level thanks to MALDI-TOF MS. Studies with higher statistical power could identify other microbial candidates, and *T. ramosa* is very likely part of a group of liver cancer oncobionts. Future studies could use deep sequencing and automated microbial culturomics [40] to focus on a larger sample to obtain a better match or at least larger groups, allowing for the stratification of all comorbidities and the stage of liver disease. Robustness is also confirmed by the fact that we found that *T. ramosa* was the most different in terms of culturomics between ALD and controls after analysis of 21 samples and 32,088 colonies, and the most significantly associated with liver cancer by DNA sequencing after analysis of 59 samples and 2,840,773 reads.

Even if future studies with larger sample size and better representation of participant groups according to the comorbidities, cause, and stage of liver disease are necessary to confirm the association and the potential role of *T. ramosa* in hepatocellular carcinoma, we believe that our results and recent literature [37] evidenced that *T. ramosa* could be a potential candidate among the gut commensals favored by alcoholism, and which can then contribute to liver carcinogenesis through its recently demonstrated genotoxic and tumor growth-promoting properties.

Abbreviations

ALD	Alcoholic liver disease
HCC	Hepatocellular carcinoma

MASH Metabolic dysfunction-associated steatohepatitis
NGS Next-generation sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-025-00703-6.

Supplementary Material 1: Supplementary methods and results

Supplementary Material 2: Culturomics results

Supplementary Material 3: 16S amplicon sequencing results

Acknowledgements
We thank Vincent Bossi for their excellent technical assistance.

Author contributions
Conceptualization, funding acquisition, and Project administration: MM, RG. Methodology and visualization: RMW, MM. Resources: AG, PB. Investigation: RMW, AA, BM, AC, CA, GM. Data curation and formal analysis: RMW, AG, AL. Supervision: MTA, MM, RG. Validation: RWM, MM, RG. Writing - original draft: RMW– review & editing: AG, MTA, MM, RG. Reviewed the results and approved the final version of the manuscript: all authors.

Funding
This work was funded by the Agence Nationale de la Recherche under two programs: ANR-15-CE36-0004-01 and ANR "Investissements d'avenir", Méditerranée Infection 10-IAHU-03. The Région Provence-Alpes-Côte d'Azur also supported this study, which received financial support from the Fondation Méditerranée Infection.

Data availability
The raw sequencing data of fecal samples are available in the NCBI Sequence Read Archive with accession number PRJEB62828. The genome of six strains of ***Thomasclavelia ramosa*** (strains CSUR Q9705, Q9779, Q9849, QA0117, QA0118, and QA0666, available on request) have been sequenced, and genome sequencing data are publicly available under the Bioproject NCBI PRJEB76822.

Declarations

Ethics approval and consent to participate
The HEPATGUT study was approved by the local ethics committee of the Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France (IHUMI, 2020-004), approved by the Protection of Persons Committee (Approval No. CPP: 21.04391.000046—21075), and carried out according to the 2013 Declaration of Helsinki (World Medical Association, 2013). Patient consent (non-opposition) was obtained according to French regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Conflict of interest
No conflict of interest is to be declared.

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Received: 8 September 2024 / Accepted: 21 April 2025
Published online: 07 May 2025

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