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THE EFFECT OF HEAT-TREATED *AKKERMANSIA MUNICIPHILA* MucT ON HEPATIC CELL MODEL OF HEPATOCELLULAR CARCINOMA

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List of abbreviations in the order of appearance:

HCC — Hepatocellular Carcinoma

CFU — Colony-Forming Units

MTT — 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

mTOR — mammalian Target of Rapamycin

mTORC — mTOR Complex

LPS — Lipopolysaccharide

LTA — Lipoteichoic acid

TLR2/TLR4 — Toll-Like Receptor 2 / Toll-Like Receptor 4

HSCs — Hepatic Stellate Cells

TNF-α — Tumor Necrosis Factor alpha

IL-6 — Interleukin-6

Tregs — Regulatory T cells

Th1 — T helper 1 cells

mMDSCs — monocytic Myeloid-Derived Suppressor Cells

A. muciniphila — Akkermansia muciniphila

RNF181 — RING Finger Protein 181

ATG7 — Autophagy-Related protein 7

JAK/STAT — Janus Kinase / Signal Transducer and Activator of Transcription

CD8+ — CD8-positive T cells

TGF-β — Transforming Growth Factor beta

IFN-γ — Interferon-gamma

ATCC — American Type Culture Collection

STR — Short Tandem Repeat

DMEM — Dulbecco's Modified Eagle Medium

FBS — Fetal Bovine Serum

MEME — Minimum Essential Medium Eagle

NEAA — Non-Essential Amino Acids

CTR — Control

DMSO — Dimethyl Sulfoxide

PBS — Phosphate-Buffered Saline

BSA — Bovine Serum Albumin

SDS — Sodium Dodecyl Sulfate

ANOVA — Analysis of Variance

SEM — Standard Error of the Mean

EdU — 5-ethynyl-2′-deoxyuridine

BrdU — 5-bromo-2′-deoxyuridine

ROS — Reactive Oxygen Species

PARP — Poly(ADP-ribose) Polymerase

Abstract

Background: Dysbiosis, through the gut–liver axis, can influence the pathogenesis of hepatocellular carcinoma (HCC). Recently, *Akkermansia muciniphila* has been shown to play a significant protective role in the development of HCC.

Aim: To assess the effects of heat-treated *A. muciniphila* MucT on cell viability and autophagy/apoptosis signaling in HCC *in vitro* models.

Methods: Murine Hepa 1-6 and NMuLi and human HepG2 cells were exposed to *A. muciniphila* (10^3 or 10 CFU) for 12–72 h after which cell viability was determined by MTT and samples were collected to assess the expression of the proteins of interest by Western blot (p-mTOR/mTOR, p70S6K, S6, Beclin-1, p62, Caspase-3).

Results: *A. muciniphila* at 10^3 CFU significantly reduced viability in Hepa 1-6 (24–48 h) and HepG2 (12–48 h); NMuLi showed an early drop at 12 h that faded by 48 h. In Hepa 1-6, p-mTOR and downstream effectors (p70S6K, S6) decreased, consistent with mTORC1 inhibition and autophagy engagement, despite a concurrent Beclin-1 reduction. While no changes were observed in mTOR-pathway markers and Beclin-1, an increase in Caspase-3 expression was found in NMuLi cells, suggesting apoptosis as the main driver of reduced viability. In HepG2, p-mTOR/mTOR rose slightly while p-p70/p70 and p-S6/S6 decreased.

Conclusions: Heat-treated *A. muciniphila* MucT directly modulates hepatic tumor cell viability and key nodes of pathways related to autophagy/apoptosis, in a cell line-specific manner. These findings support the potential usefulness of *A. muciniphila* as an adjuvant strategy to improve treatment response in HCC patients.

Background

Hepatocellular carcinoma (HCC) is the sixth most common malignancy world-wide, being the most common primary liver malignancy [1]. Pathogenesis is complex and multifactorial, while numerous aspects could influence tumor growth and development. However, the potential role of the gut microbiome in this context is of growing interest, while remaining in an exploratory stage.

The role of gut microbiome

Dysbiosis leads to the condition known as “leaky gut”, characterized by increased intestinal permeability that facilitates the translocation of microbial products such as Lipopolysaccharide or Lipoteichoic acid (LPS/LTA).

The leaky gut is present in a wide range of diseases, including patients with advanced chronic liver disease as compared to healthy people [2]. Specifically, cirrhotic patients present a lower rate of butyrate-producing species, considered as beneficial, like *Coprococcus spp.*, *Faecalibacterium prausnitzii*, and members of the *Lachnospiraceae* and an overgrowth of *Ruminococcaceae*, with *Veillonella*, *Streptococcus*, and *Clostridium perfringens* [3]. Moreover, *Alphaproteobacteria* and *Verrucomicrobia* phylum were significantly reduced in cirrhotic patients with concomitant HCC compared to those without malignancy [4]. Indeed, patients with liver cirrhosis and HCC show specific patterns of dysbiosis, characterized by the overrepresentation of pro-inflammatory and pathogenic bacteria (such as *Enterobacter, Enterococcus, Klebsiella*) together with decreased beneficial bacteria (like *Akkermansia, Bifidobacterium*, and butyrate-producing species) [5].

Regarding the pathophysiological standpoint, when microbial products reach the liver, they activate Toll-Like Receptor 2 / Toll-Like Receptor 4 (TLR2/TLR4) on hepatic immune and stromal cells i.e. hepatic stellate cells (HSCs) and trigger Tumor Necrosis Factor alpha / Interleuchin-6 (TNF-α/IL-6)–driven chronic inflammation and fibrogenesis with an immunotolerant shift, increasing the infiltration of Regulatory T cells (T_{regs}), while reducing the presence of T helper 1 (Th1) cells. Preclinical models show microbiota–TLR4 signaling promotes cirrhosis/HCC by expanding monocytic myeloid-derived suppressor cells (mMDSCs), inducing a pro-inflammatory setting and immune tolerance which translate into a protumor environment [6-7]

Among the microbiota components, *Akkermansia muciniphila* (*A. muciniphila*) has recently gain a great scientific interest due to its anti-inflammatory and immuno-stimulating role, which could impact on tumor growth by improving the micro-immune environment in favor of a more cytotoxic and less immunosuppressive profile [8-9]. Moreover, several studies have underscored the hepatoprotective effect of *A. muciniphila* in liver injury [10-11], and recently, authors deeply explored its role in HCC context, showing that its presence may influence both tumor growth and, interestingly, treatment response [12].

Although the mechanism underlying the effect of *A. muciniphila* on tumor cells has yet to be elucidated, some evidence from other tumors suggests that it may involve the induction of autophagic mechanisms.

Potential autophagy/apoptosis-inducing effect of A. muciniphila

Autophagy and apoptosis are two fundamental but mechanistically distinct cellular programs.

On the one hand, autophagy is a catabolic recycling process that degrades cytoplasmic components through the lysosomal system to maintain energetic and metabolic homeostasis under stress. This process is a fundamental mechanism that preserves cellular homeostasis, and alterations in this pathway contribute to the pathogenesis of multiple human disorders, including cancer. Notably, autophagy exerts highly context-dependent effects in tumor biology. In early stages of tumorigenesis, efficient autophagic clearance of damaged organelles and macromolecules can act as a tumor-suppressive mechanism by limiting genomic instability and chronic inflammation. [13-14]. The canonical autophagy signaling axis is primarily driven by nutrient/energy sensing pathways converging on the central kinase mechanistic target of rapamycin (mTOR) [15]. Under nutrient-replete status, mTORC1 actively suppresses autophagy initiation by phosphorylating downstream effectors such as p70 S6 kinase and S6 and, thus, promoting anabolic growth. In this context, phosphorylation of signaling proteins (e.g. phospho-mTOR, phospho-p70 S6K, phospho-S6) is a critical readout, because phosphorylation is the biochemical "ON/OFF" switch that modulates their activity and integrates growth signals [16-17]. When nutrients, ATP or amino acids drop, mTORC1 becomes inhibited, which releases the ULK1-ATG13-FIP200 complex and enables autophagosome biogenesis. In contrast, Beclin-1 is a core pro-autophagic scaffold required for autophagosome nucleation and therefore is essential for autophagy initiation and progression. p62 is a selective autophagy cargo receptor that binds ubiquitinated cargo and is itself degraded inside autolysosomes – therefore p62

accumulation is a strong indicator of impaired autophagic flux, whereas p62 reduction typically indicates active, functional autophagy [13, 18].

On the other hand, apoptosis is a highly regulated form of programmed cell death that eliminates damaged or unwanted cells. The effector caspase-3 is the protease that executes the final dismantling of the cell by cleaving multiple structural and regulatory substrates. Importantly, caspase-3 is commonly used as a marker of apoptotic commitment [19].

The closest evidence that points towards autophagy modulation as the main mechanism of *A. muciniphila* to prevent tumor growth comprises the increased expression levels of RING Finger Protein 181 (RNF181), which ubiquitinates Autophagy-Related protein 7 (ATG7), a key autophagy protein seen in lung adenocarcinoma cells after incubation with *A. muciniphila* outer membrane protein component Amuc_1100 [20]. Inhibition of Janus Kinase / Signal Transducer and Activator of Transcription (JAK/STAT) pathway, which prevents immune escape and enhances CD8-positive (CD8+) T cell recruitment [21], suppresses Transforming Growth Factor beta (TGF-β) signaling and promotes tumoral cells apoptosis [22] was also found in an *in vitro* model of this type of tumor.

Moreover, other studies have described the effect of Amuc proteins in other digestive tumors, such as colorectal and gastric cancer. For example, protein Amuc_1434 was able to restore cytotoxic activity in colorectal cancer, triggering antitumor immune response [23-24]. Amuc proteins could also induce apoptosis in gastric cancer cells and modulate the behavior of the immune population within the tumor microenvironment. Specifically, these proteins promoted macrophage polarization towards M1-like phenotype (typically associated with antitumor response and

inflammation) and increased cytotoxic-related cytokines (e.g., Interferon-gamma IFN-γ), while suppressing T_{reg}-related cytokines [25].

Given the qualities of *A. muciniphila* and its impact on tumor growth, there is a need to evaluated its use as probiotic supplementation in HCC patients, gathering evidence from in preclinical models to then translate results into the clinics. The proposed type of formulation could provide a potential added value compared to live strain formulations [26].

Aim

The main aim of the study is to explore the potential usefulness of *A. muciniphila*-based probiotic supplementation to prevent tumor growth in HCC using *in vitro* models.

For this pruropse, the following partial aims were established:

1) to evaluate *in vitro* the effects of heat-treated *A. muciniphila on cell viability*, using HCC cell lines models and a non-tumor cell line,
2) to explore the possible *A. muciniphila-mediated* modulation of autophagic and apoptotic pathways in tumor cells by studying the main proteins involved.

Material and Methods

Cell lines

For this study, Hepa 1-6 (murine cells, American Type Culture Collection ATCC cat. no. CRL-1830), NMuLi (murine cells, ATCC cat. no. CRL-1638), and HepG2 (human cells, ATCC cat. no. HB-8065) cell lines were used. On the one hand, Hepa 1-6 cell line is a murine hepatocellular carcinoma line, which expresses several cytochrome P450 enzymes [27].On the other hand, HepG2 is a human cell line, widely used in liver research as an *in vitro* model of human hepatocytes, although they are immortalized cells derived from young hepatocellular carcinoma patient and, as such, they exhibit dedifferentiated traits such as reduced expression of certain metabolic functions compared with primary human hepatocytes [28]. Lastly, murine NMuLi cell line is an immortalized line derived from normal liver cells and therefore expresses hepatocyte-specific enzymes and genes.

All cell lines involved were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), expanded, and stored in the Cell Bank of the Cell Culture Facility at CEINGE-Advanced Biotechnology "Franco Salvatore" (Naples, Italy). Cells were periodically tested for mycoplasma (MycoAlert, Lonza; cat. no. LT07-318) and authenticated by short tandem repeat (STR) profile analysis. Cells were cultured at 37 °C in 5% CO_2. For both murine cell lines (Hepa 1-6 and NMuLi cells) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Gibco, cat. no. A5256701) and 1% (v/v) L-glutamine (Gibco, cat. no. 25030024) was used. On the other hand, HepG2 cells were cultured using Minimum Essential Medium Eagle (MEME; Sigma-Merck, cat. no. M2279) with 10% (v/v) FBS (Gibco), 1% (v/v) L-glutamine (Gibco), and 1% Non-Essential Amino Acids solution (NEAA; Sigma-Merck, cat. no. M7145), due to their human nature.

Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [29]. This colorimetric assay is based on the ability of viable cells to metabolize MTT (yellow) into formazan (blue/purple) by mitochondrial enzymes.

Hepa 1-6, NMuLi, and HepG2 cells (5×10^3 cells/well) were plated in 96-well plates in complete medium for 24 h. Subsequently, they were stimulated in complete medium with 2% FBS with two different doses of heat-treated *Akkermansia muciniphila MucT* according to the following experimental groups:

- Control group (CTR**)**: Hepa 1-6 or NMuLi or HepG2 cells were incubated in 2% FBS medium
- Treatment 1: cells were incubated with *A. muciniphila* 10^3 Colony-Forming Units (CFU) in 2% FBS medium
- Treatment 2: Hepa 1-6 or NMuLi or HepG2 cells were incubated with *A. muciniphila* 10^1 CFU in 2% FBS medium

All dilutions were prepared in antibiotic-free 2% FBS complete medium, following serial dilutions (starting from the initial concentration reported in the specific section of the datasheet supplied with the product). After 12, 24, 48, and 72 h of incubation at 37 °C, MTT (Sigma–Merck) was added to the medium to a final concentration of 0.5 mg/mL. After 1h-incubation in the dark at 37 °C, the resulting formazan salts were dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured (EnSpire Multimode Plate Reader, PerkinElmer, Waltham, MA, USA). The optical density of wells treated with the different concentrations of *A. muciniphila* (OD_{480}) was compared with the CTR OD_{480}. Cell viability for each experimental group was calculated as:

$$\% \text{ viability} = (\text{treated OD} / \text{control OD}) \times 100$$

Western blot Analysis

Hepa 1-6, NMuLi, and HepG2 cell lines (5×10^3 cells/well) were plated in 6-well plates in complete medium for 24 h. Subsequently, cells were stimulated in antibiotic-free complete medium with 2% FBS in the presence of heat-treated *A. muciniphila* for 24 h. To obtain cell lysates for western blotting analysis of the proteins of interest. Cells were homogenized on ice with cold lysis buffer (20 mM Tris-HCl, pH 7.5; 10 mM NaF; 150 mM NaCl; 1% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; 1 mM Na_3VO_4; leupeptin and trypsin inhibitor 10 µg/mL). After 1 h, samples were centrifuged at 12,000 rpm for 20 min at 4 °C to obtain the lysates. Protein concentration was determined using the Bio-Rad protein assay and bovine serum albumin (BSA) as the standard.

Lysates (40 µg) were dissolved in Laemmli sample buffer, heated at 95 °C for 5 min, loaded onto Sodium Dodecyl Sulfate (SDS)-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes using the TransBlot Turbo (Bio-Rad Technologies, Milan, Italy). Membranes were blocked with 1× phosphate-buffered saline (PBS) containing 3% non-fat dried milk for 45 min at room temperature, then incubated with the following primary antibodies: anti-phospho-mTOR (mammalian Target of Rapamycin) (1:1000; cat. no. 2971, Cell Signaling), anti-mTOR (1:1000; cat. no. 2983, Cell Signaling), anti-phospho-S6 (1:1000; cat. no. 2215, Cell Signaling), anti-S6 (1:1000; cat. no. 2217, Cell Signaling), anti-phospho-p70 S6 kinase (1:1000; cat. no. 9205, Cell Signaling), anti-p70 S6 kinase (1:1000; cat. no. 9202, Cell Signaling), anti-Beclin 1 (1:1000; cat. no. sc-48341, Santa Cruz

Biotechnology), anti-p62 (1:1000; cat. no. ab56416, Abcam), and anti-Caspase-3 (1:1000; cat. no. 9662, Cell Signaling). All antibodies were diluted 1:1000 in 1× PBS with 3% non-fat dried milk and 0.1% Tween-20. Membranes were incubated at 4°C overnight, then with the corresponding secondary antibody for 1 h at room temperature. Chemiluminescence was detected using the ChemiDoc Imaging system (Bio-Rad Laboratories, Hercules, CA, USA). To confirm equal loading, membranes were also probed with anti-β-actin (1:10·000; cat. no. A5441, Sigma-Merck).

Statistical analysis

Data were reported as mean ± Standard Error of the Mean (SEM: SD /√n) and used for one-way analysis of variance (ANOVA) for multiple comparisons, followed by Bonferroni's post hoc test. GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for these analyses. Differences between experimental groups were considered significant at $p < 0.05$.

Results

Akkermansia muciniphila modulated cell viability in a cell line-dependent manner

A. muciniphila influenced cell viability, this effect was clearly dose-dependent and differed across cell lines. The lowest dose (10 CFU) had no measurable impact on viability at any time point in any of the tested models (Figure 1). In Hepa 1-6 cells, the highest dose (10^3 CFU) significantly reduced viability at 24 h and 48 h (Fig. 1B and 1C, ****$p < 0.0001$ vs CTR), whereas no effect was detected at 12 h (Fig. 1A). In the normal murine NMuLi cell line, a significant reduction in viability was already detectable at 12 h at the same dose (Fig. 1D and 1E, ****$p < 0.0001$ *vs* CTR), while this difference was no longer present at 48 h (Fig. 1F). Finally, in the human tumor HepG2 cells, viability was markedly reduced as early as 12 h and remained reduced up to 48 h (Fig. 1G, 1H, and 1I, ****$p < 0.0001$ vs CTR).

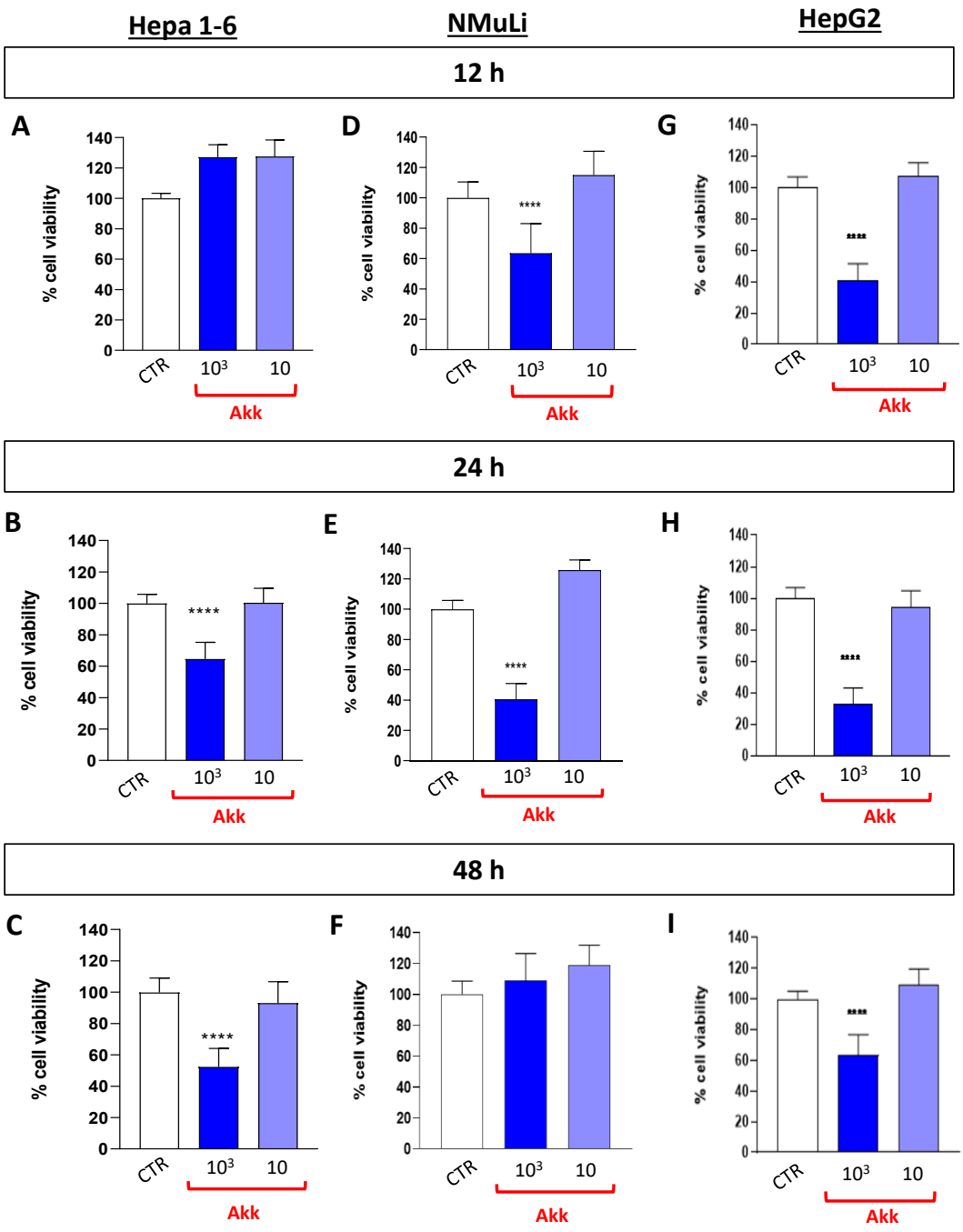

Figure 1. Effects on cell viability.

Effect of *Akkermansia muciniphila* MucT heat-treated (Akk; 10^3 and 10 CFU) on cell viability (12, 24, 48 h) in the following cell lines: A–C) Hepa 1-6 (murine hepatic tumor cells), D–F) NMuLi (normal murine hepatic cells), and G–I) HepG2 (human hepatic tumor cells). After incubation with Akk, cell viability was assessed by the MTT assay (5×10^3 cells/well; n = 6). The assay was performed in triplicate for each experimental time point. Data is shown as mean ± SEM; ****$p < 0.0001$ vs CTR (one-way ANOVA followed by Bonferroni's post hoc test).

Akkermansia muciniphila modulated autophagy-related markers in Hepa 1-6, NMuLi and HepG2

Since the highest dose of *A. muciniphila* (10^3 CFU) significantly reduced cell viability in all lines after 24 h, we have selected this incubation time to perform the following studies aimed at understanding the mechanisms underlying this effect observed in all three cell lines. Given the possible involvement of cellular protective strategies such as activation of autophagic and apoptotic pathways in reducing cell viability after being exposed to *A. muciniphila* we performed an expression analysis of known effectors of these pathways in all three cell lines (Figures 2-5).

In murine tumor Hepa 1-6 cells the highest dose of *A. muciniphila* (10^3 CFU) induced a significant reduction in mTOR phosphorylation (p-mTOR/mTOR ratio; Fig. 2B) and in all of its downstream effectors at 24 h (Fig. 2C, 2D, 2F), suggesting its involvement of the autophagic pathway. Unexpectedly, this might appear inconsistent with the concomitant reduction in Beclin-1 expression (Fig. 2E); however, this protein is also involved in other complex and balanced cellular pathways (e.g. protein–protein interactions, phosphorylations, ubiquitinations, and cleavages) making it a point of convergence between autophagy, apoptosis, and cell proliferation

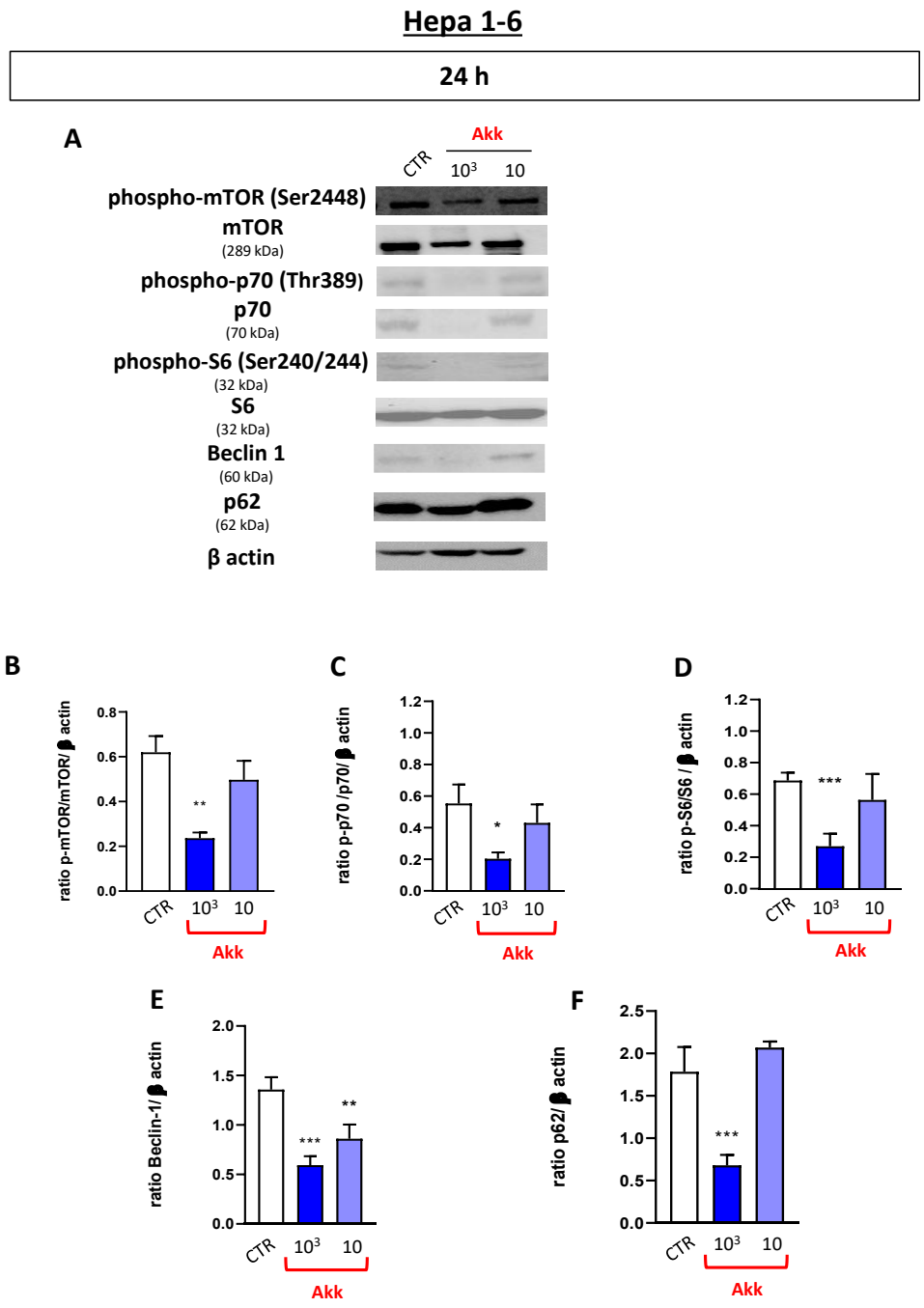

Figure 2. Protein expression analysis of the main factors involved cellular autophagic and apoptotic mechanisms in Hepa 1-6 cells.

Protein expression levels of phospho-mTOR, mTOR, phospho-p70 S6 kinase, p70 S6 kinase, phospho-S6, S6, Beclin-1, and p62 in Hepa 1-6 cells after being incubated for 24 h with heat-treated *Akkermansia muciniphila* MucT (Akk). A) Representative blots; B–F) densitometric analyses of the examined proteins. Experiments were performed in triplicate. Densitometry is expressed as arbitrary units. Values are reported as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs CTR; one-way ANOVA followed by Bonferroni's post hoc test).

Otherwise, in normal murine NMuLi cells, the autophagic pathway did not appear to be activated, since the highest dose of *A. muciniphila* did not induce any significant changes in mTOR phosphorylation (p-mTOR/mTOR ratio; Fig. 3B) or in downstream proteins of its pathway (Fig. 3C, 3D, 3F) and Beclin-1 levels remained unchanged (Fig. 3E). These data suggest that the reduction in viability at 24 h in NMuLi is likely driven by alternative mechanisms. Regarding HepG2 cell lines, the highest dose of *A. muciniphila* (10^3 CFU) slightly increased the p-mTOR/mTOR ratio (Fig. 4B), while the phosphorylated-to-total protein ratios of both p70 and S6 were reduced (Fig. 4C, 4D). The expression of the other proteins examined remained unchanged (Fig. 4E, 4F).

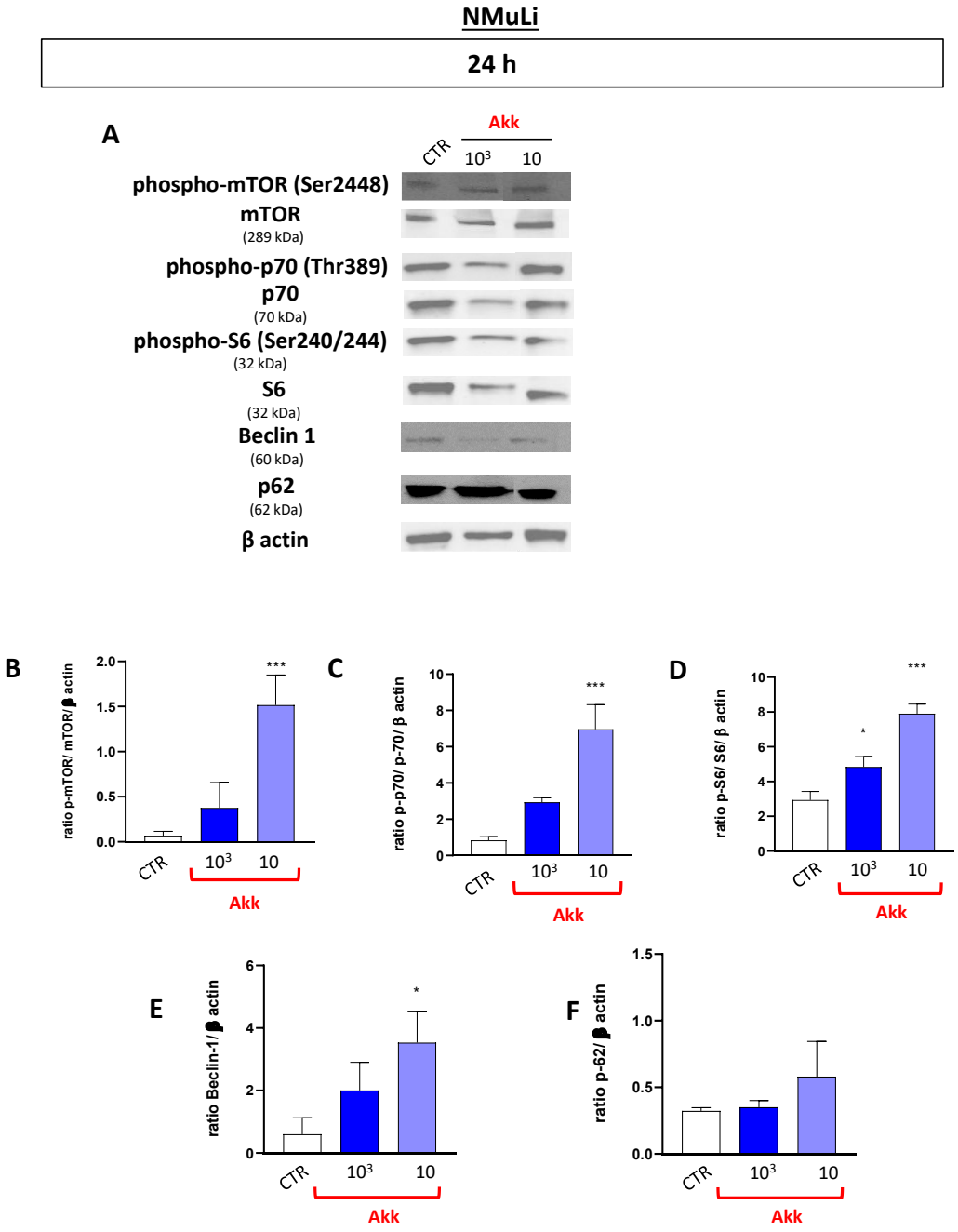

Figure 3. Protein expression analysis of the main factors involved cellular autophagic and apoptotic mechanisms in NMuLi cells.

Protein expression levels of phospho-mTOR, mTOR, phospho-p70 S6 kinase, p70 S6 kinase, phospho-S6, S6, Beclin-1, and p62 in NMuLi cells after being incubated for 24 h with heat-treated *Akkermansia muciniphila* MucT (Akk). A) Representative blots; B–F) densitometric analyses of the examined proteins. Experiments were performed in triplicate. Densitometry is expressed as arbitrary units. Values are reported as mean ± SEM (*p<0.05 and ***p<0.001 vs CTR; one-way ANOVA followed by Bonferroni's post hoc test).

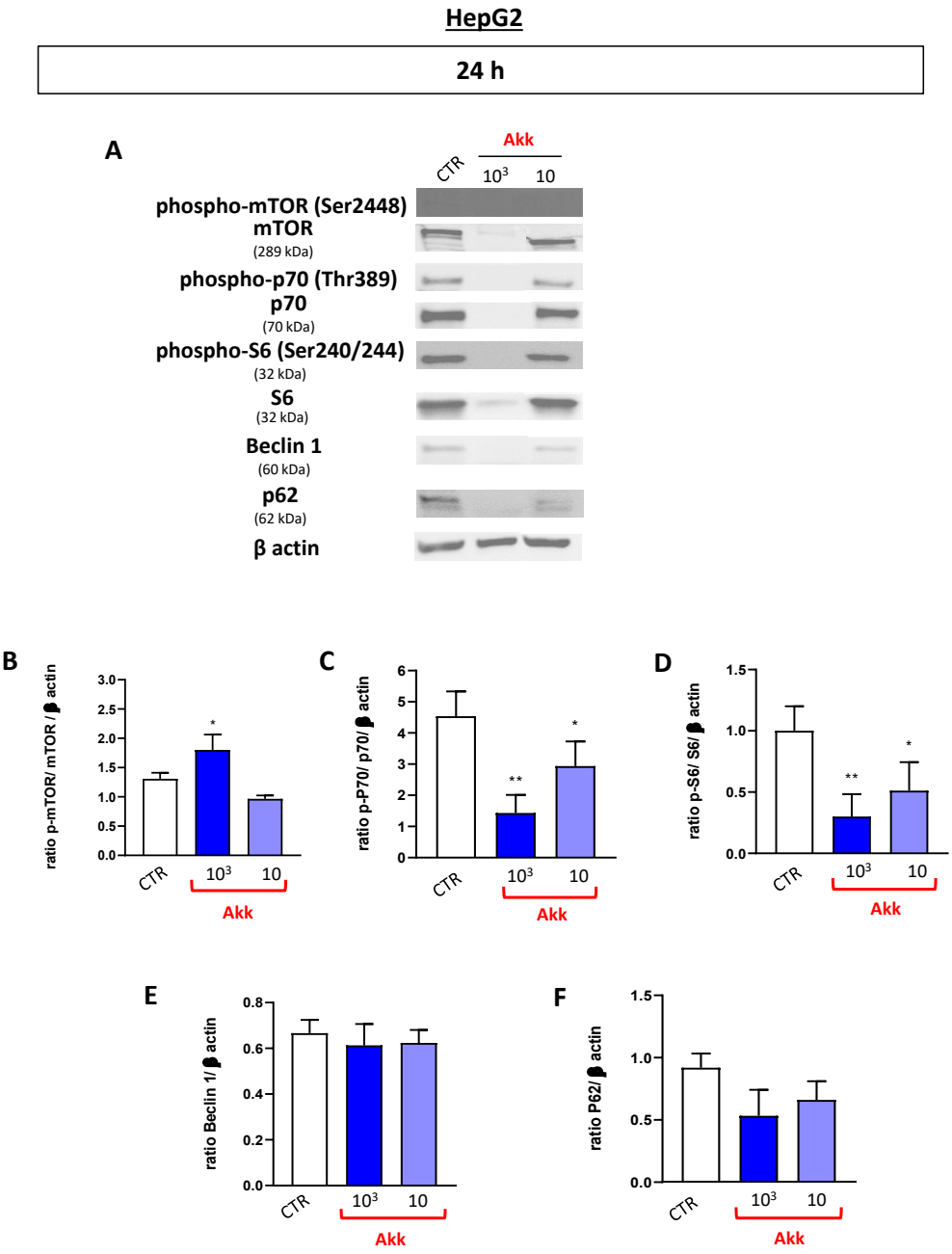

Figure 4. Protein expression analysis of the main factors involved cellular autophagic and apoptotic mechanisms in HepG2 cells.

Protein expression levels of phospho-mTOR, mTOR, phospho-p70 S6 kinase, p70 S6 kinase, phospho-S6, S6, Beclin-1, and p62 in the HepG2 cell line after being incubated for 24 h with heat-treated *Akkermansia muciniphila* MucT (Akk). A) Representative blots; B–F) densitometric analyses of the examined proteins. Experiments were performed in triplicate. Densitometry is expressed as arbitrary units. Values are reported as mean ± SEM (*p<0.05 and **p<0.01 vs CTR; one-way ANOVA followed by Bonferroni's post hoc test).

Akkermansia muciniphila influenced the expression of the apoptotic protein Caspase-3 in a cell line–specific manner

Lastly, we assessed Caspase-3 expression (Fig. 5), a protein involved in the two cellular apoptotic signaling pathways. In Hepa 1-6, Caspase-3 was not significantly modified by any dose (Fig. 5B). In contrast, Caspase-3 levels were significantly increased in NMuLi, consistent with activation of apoptotic signaling in this non-tumor line (Fig. 5D). Conversely, HepG2 cells showed a significant reduction in Caspase-3 at the highest dose (Fig. 5F), highlighting that the autophagy–apoptosis interplay in this human tumor model is more complex and likely involves additional regulatory layers.

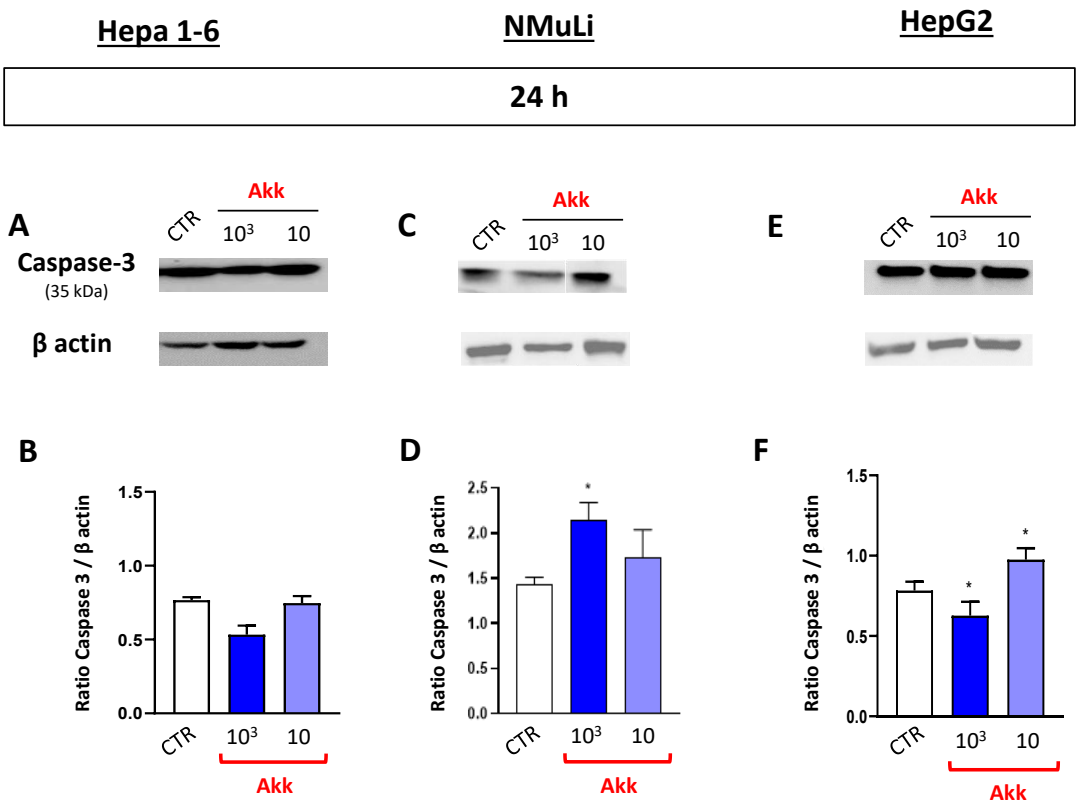

Figure 5. Analysis of Caspase-3 expression in hepatic cell lines.

24-h effect of heat-treated *Akkermansia muciniphila* MucT (*Akk*) on Caspase-3 expression in Hepa 1-6 (murine hepatic tumor), NMuLi (normal murine), and HepG2 (human hepatic tumor) cell lines. A–C–E) Representative blots; B–D–F) densitometric analyses of the examined proteins. Experiments were performed in triplicate. Densitometry is expressed as arbitrary units. Values are reported as mean ± SEM (*p<0.05 vs CTR; one-way ANOVA followed by Bonferroni's post hoc test).

Discussion

Overall, our results indicate that the heat-treated *A. muciniphila* formulation reduces cell viability in a time- and dose-dependent manner in Hepa 1-6 and HepG2 cell lines, whereas in NMuLi the effect is earlier and transient. Moreover, the strongest and most sustained viability-reducing effects were observed in the tumorigenic Hepa 1-6 and HepG2 models, while the impact in non-transformed NMuLi cells was limited in magnitude and transient. This finding is particularly relevant from a translational point of view: it suggests a degree of tumor selectivity and, therefore, supports the potential use of heat-treated *A. muciniphila* (or its bioactive components) as an adjunct or adjuvant strategy to selectively stress malignant hepatocytes while sparing normal liver tissue. Mechanistically, our data support that these viability changes involve distinct cell death programs depending on the cellular context, due to the relationship between autophagy and apoptosis, depending on triggers [30-31]. Notably, since this study uses a heat-treated preparation, the biological activity observed here falls under the emerging "postbiotic" concept. Postbiotics are considered inherently safer than live bacteria, which increases their translational feasibility for cancer patients, who often present with mucosal barrier dysfunction and immunosuppression. In Hepa 1-6, the decrease in p-mTOR and its downstream effectors at 24 h strongly suggests inhibition of mTORC1 signaling and engagement of the autophagic program. The concomitant reduction in Beclin-1 is not necessarily contradictory: Beclin-1 is a highly pleiotropic scaffold integrating multiple post-translational regulatory events (PTMs), and its protein abundance alone does not directly report autophagic flux. According to field guidelines, lysosomal blockade assays are therefore needed for an interpretable autophagy readout [32-35].

In NMuLi, the absence of mTOR-pathway changes alongside an increase in Caspase-3 expression supports an apoptotic response as the main mechanism driving the transient reduction in viability, while autophagic pathway is negligible in these cell lines.

In HepG2, the marginal increase of the p-mTOR/mTOR ratio is accompanied by a decreases in p-p70/p70 and p-S6/S6 ratio. highlights a non-linear cross-talk between metabolic signaling and stress pathways; this type of "mixed" signature is in line with the concept that tumor cells can engage hybrid stress responses with partial autophagy and partial apoptosis depending on their oncogenic wiring. Importantly, in the broader context of cancer biology, these findings are relevant because autophagy manipulation is now recognized as a potential vulnerability in HCC. Our work provides initial evidence that heat-treated *A. muciniphila* is not merely "pro-autophagy" in all contexts but can selectively trigger distinct death programs in different hepatic cell models. To our knowledge, this is the first study demonstrating an *in vitro* antitumor effect of *A. muciniphila* alone in an HCC setting, suggesting that microbial components could be exploited as metabolic modulators of stress pathways in cancer cells. Finally, if heat-treated *A. muciniphila* selectively stresses tumor cells via metabolic signaling rewiring, this could increase their sensitivity to existing systemic therapies (e.g. kinase inhibitors or immunotherapy). Future studies should therefore evaluate whether postbiotic priming could potentiate existing HCC regimens.

Although these promising findings, several limitations merit consideration when interpreting our *in vitro* model. Our experiments used a single heat-treated formulation, *in vitro* monocultures, and limited time points (mainly 24-48 h), which limits mechanistic inference and clinical translation. The absence of an immune compartment is particularly relevant in HCC, where immunity and hepatic microbiome

cross-talk are critical determinants of treatment response. It is therefore plausible that *in vivo* a substantial fraction of the antitumor effect could be mediated indirectly through immune or stromal modulation (e.g. macrophage polarization, DC activation, modulation of hepatic stellate cell functions), which is not captured in 2D monocultures.

To strengthen our findings, future work should: (i) demonstrate autophagic flux; (ii) refining apoptosis readouts (cleaved Caspase-3/7, Poly(ADP-ribose) Polymerase PARP cleavage, Annexin V/Propidium Iodide) and cell-cycle analyses; (iii) mapping upstream/downstream steps to clarify the divergent patterns in HepG2; (iv) extending to additional HCC lines (Huh7, PLC/PRF/5, SNU-449) and functional assays (clonogenic survival, 5-ethynyl-2′-deoxyuridine / 5-bromo-2′-deoxyuridine EdU/BrdU, migration/invasion, Reactive Oxygen Species ROS) to distinguish cytotoxic from cytostatic effects.

Finally, if heat-treated *A. muciniphila* selectively rewires metabolic-stress pathways in tumor cells, it might increase their susceptibility to existing systemic HCC treatments (e.g. targeted kinase inhibitors or immunotherapy), raising the possibility of using postbiotic priming as a therapeutic sensitizer.

Conclusions

In conclusion, our *in vitro* models have demonstrated the efficacy of heat-treated *Akkermansia muciniphila* in inhibiting hepatocellular carcinoma cell lines, representing a milestone toward a broader understanding of the gut microbiome role in the biology of tumorigenesis.

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