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► **To cite this version:**

Rodolfo Garza-Morales, Beatriz Rendon, Mohammad Tariq Malik, Jeannete Garza-Cabrales, Anne Aucouturier, et al.. Targeting Melanoma Hypoxia with the Food-Grade Lactic Acid Bacterium *Lactococcus Lactis*. *Cancers*, 2020, 12 (2), pp.438. 10.3390/cancers12020438 . hal-04565131

HAL Id: hal-04565131

<https://hal.inrae.fr/hal-04565131v1>

Submitted on 11 Sep 2024

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Article

Targeting Melanoma Hypoxia with the Food-Grade Lactic Acid Bacterium *Lactococcus Lactis*

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Received: 27 December 2019; Accepted: 10 February 2020; Published: 13 February 2020



Abstract: Melanoma is the most aggressive form of skin cancer. Hypoxia is a feature of the tumor microenvironment that reduces efficacy of immuno- and chemotherapies, resulting in poor clinical outcomes. *Lactococcus lactis* is a facultative anaerobic gram-positive lactic acid bacterium (LAB) that is Generally Recognized as Safe (GRAS). Recently, the use of LAB as a delivery vehicle has emerged as an alternative strategy to deliver therapeutic molecules; therefore, we investigated whether *L. lactis* can target and localize within melanoma hypoxic niches. To simulate hypoxic conditions *in vitro*, melanoma cells A2058, A375 and MeWo were cultured in a chamber with a gas mixture of 5% CO₂, 94% N₂ and 1% O₂. Among the cell lines tested, MeWo cells displayed greater survival rates when compared to A2058 and A375 cells. Co-cultures of *L. lactis* expressing GFP or mCherry and MeWo cells revealed that *L. lactis* efficiently express the transgenes under hypoxic conditions. Moreover, multispectral optoacoustic tomography (MSOT), and near infrared (NIR) imaging of tumor-bearing BALB/c mice revealed that the intravenous injection of either *L. lactis* expressing β -galactosidase (β -gal) or infrared fluorescent protein (IRFP713) results in the establishment of the recombinant bacteria within tumor hypoxic niches. Overall, our data suggest that *L. lactis* represents an alternative strategy to target and deliver therapeutic molecules into the tumor hypoxic microenvironment.

Keywords: *Lactococcus lactis*; probiotic; hypoxia; melanoma; tumor microenvironment; MSOT; advance molecular imaging (AMI), targeted therapies; solid tumors

1. Introduction

Melanoma is the most aggressive form of skin cancer and, if disseminated through the dermis, has a poor prognosis and high mortality rate. There are various biological mechanisms that affect melanoma treatment such as epigenetic modifications, tumor heterogeneity, tumor plasticity, interactions within the tumor microenvironment, among others [1].

The tumor microenvironment is a highly heterogeneous and complex landscape where interactions between malignant and non-malignant cells play an important role in cancer development, spread, and

response to treatment [2,3]. An important feature of the tumor microenvironment is hypoxia, this factor is associated with advanced stages of malignancy and negatively affects the response to treatment by making drug diffusion to tumor sites difficult [4–6]. Studies have shown that current therapeutic approaches are unable to target tumor cells in poorly perfused areas due to a deficient vasculature [7–9]. Moreover, the hypoxic tumor region has a pivotal role in the development of multidrug resistance to anticancer drugs; immune response suppression; increased metastasis; and increased risk of mortality, independently of prognostic factors such as grade, histology, nodal status and size of the tumor [10–13]. Therefore, selectively targeting the hypoxic tumor microenvironment represents an attractive strategy for melanoma treatment.

Hypoxia, a reduction in the oxygen tension of organs, tissues, or cells is caused by an imbalance between oxygen supply and consumption [14]. Hypoxia is a hallmark of solid tumor formation and a potent driver of the malignant phenotype [15]. Through both redox effects and the stabilization of hypoxia inducible factors (HIFs), hypoxia can influence tumorigenesis and the maintenance of the tumor microenvironment [16]. Therefore, a strategy to take advantage of the hypoxic microenvironment is through the preferential accumulation of anaerobic bacteria into tumor hypoxic niches [17–21].

The desirable characteristics for using facultative anaerobic bacteria as a cancer-targeting gene delivery vehicle are that: (1) they are self-propelled and penetrate into poorly vascularized tissue regions; (2) they have the ability to target hypoxic regions to improve tumor specificity; and (3) they are amenable to genetic manipulation [22]. Although the initial focus of using bacteria as an anti-cancer delivery vehicle has centered on *Salmonella*, *Listeria*, and *Clostridium*, the inherent risk of reversion to a virulent strain via gene transfer or mutation substantially limits their application in a clinical setting [23]. The use of probiotic bacteria as anti-cancer delivery vehicle represents an attractive strategy to circumvent the risk of reversion of attenuated strains. *Lactococcus lactis* (*L. lactis*) is a gram-positive facultative anaerobic lactic acid bacterium (LAB) generally recognized as safe (GRAS) according to the United States Food and Drug Administration (FDA). Recently, the use of LAB as a delivery vehicle has emerged as an attractive strategy to deliver a diverse range of therapeutic molecules for applications in allergic, infectious, gastrointestinal diseases and cancer [24–26]. Using an inducible gene expression system prevents systemic drug delivery and side effects. This control over gene expression can deliver therapeutic molecules specifically at the tumor microenvironment. The most widely used protein expression system for *L. lactis* is the NICE (Nisin Controlled Gene Expression) system in which the expression of the gene of interest is tightly regulated, and high expression levels are achieved using nisin, a food-grade molecule, as an inducer [27]. Recently, another inducible expression system was described, the Stress-Inducible Controlled Expression (SICE) system, which uses a promoter of the *L. lactis* *groESL* operon, and whose expression is induced under stress conditions, which does not need exogenous induction, nor the presence of regulatory genes [28].

Expression of fluorescent reporter genes by bacteria is used for many applications including gene expression analysis and imaging in vivo. We recently developed *L. lactis* encoding green fluorescent protein (GFP) and mCherry under the NICE system, *L. lactis* was able to efficiently express these reporter proteins, which were easily visualized by fluorescent microscopy [29]. Multispectral optoacoustic tomography (MSOT) is a unique, high-resolution, non-invasive in vivo imaging technique that resolves optical contrast, but the resolution obeys the rules of ultrasonic diffraction. Moreover, optoacoustic methods have the ability to render photon scattering irrelevant to image formation, enabling the capability for 3D high-resolution at depth and in real-time [30–32]. To date, optoacoustic methods can identify solid tumors, various aspects of vascularity, and track biodistribution of intravenously and orally administrated nanoparticles [31,33–36]. To the best of our knowledge, herein we report, for the first time, the use of MSOT to track *L. lactis* distribution into tumor hypoxic niches.

In this study, we aimed to investigate whether *L. lactis* can target and localize within melanoma hypoxic niches. We report that the model LAB *L. lactis* successfully expresses recombinant fluorescent proteins under hypoxic conditions both in vitro and in vivo and has the ability to target melanoma

tumors in vivo, which was detected with optoacoustic methods. *L. lactis* represents an alternative strategy to target and deliver molecules of health interest into the tumor hypoxic microenvironment.

2. Results

2.1. Effect of Cobalt Chloride and Hypoxic Conditions on Human Melanoma Cell Viability

Skin-derived melanoma cells A375 and A2058 and metastatic lymph-node derived MeWo cells were treated with increasing concentrations of cobalt chloride (CoCl_2), a chemical inducer of hypoxia-inducible factor 1 α (HIF-1 α) [37]. At 24 h post-treatment, an MTT assay revealed that melanoma cell survival decreased in a CoCl_2 dose-dependent manner. However, we detected a difference between the cell lines tested; the percentage of cell viability at 24 h post-treatment with CoCl_2 at 200 μM was 76% in A2058 cells, 27% in MeWo cells, and 36% in A375 cells. At 48 h post-treatment, the percentage of cell viability was 45% in MeWo cells, 31% in A2058 cells and 30% in A375 cells. At 72 h post-treatment, there was no significant difference in cell viability between the different cell lines tested (Figure 1a).

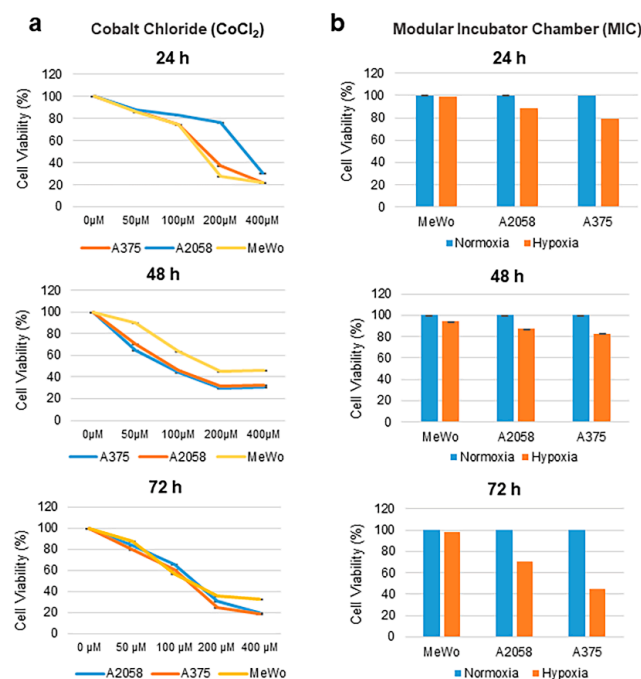


Figure 1. Effect of cobalt chloride (CoCl_2) and hypoxia on melanoma cell viability. (a) A2058, A375 and MeWo cells were treated with CoCl_2 at increasing concentrations (0, 50, 100, 200, 400 μM) for 24, 48, and 72 h. (b) A2058, A375, and MeWo cells were cultured in a modular incubator chamber (MIC) in hypoxic conditions (1% O_2 , 5% CO_2 and 94% N_2) or normoxic conditions for 24, 48 and 72 h. Cell survival was calculated by MTT assay. Results represent the mean of three repeated measurements \pm standard deviation (SD; error bars) (* $p < 0.05$).

Next, we sought to validate these results by incubating melanoma cells in normoxic or hypoxic conditions using a modular incubator chamber (MIC) [38]. The percentage of cell viability was evaluated at 24, 48, and 72 h. An MTT assay revealed that at 24 h following incubation in hypoxic conditions, A2058 and A375 cell viability decreased 12% and 22%, respectively, when compared to cells incubated in normoxic conditions. This was in contrast to MeWo cells, which were unaffected by hypoxia. At 48 h, an increase in the difference of cell viability between cells incubated in normoxic and hypoxic conditions was no longer observed. However, at 72 h, the percentage of A2058 and A375 cell viability decreased 30% and 55%, respectively, in comparison with normoxic conditions. In contrast, MeWo cells remained unaffected, as the percentage of cell viability was similar to normoxic conditions.

These results show that metastatic lymph-node derived MeWo cells have superior cell survival ability over skin-derived A2058 and A375 cells (Figure 1b).

2.2. Induction of Hypoxia-Inducible Factor 1 α (HIF-1 α) in Human Melanoma Cells

To demonstrate hypoxia induction in melanoma cells *in vitro*, HIF-1 α expression was evaluated after CoCl₂ treatment or incubation in hypoxic conditions. Cobalt chloride, a hydroxylase inhibitor, can mimic hypoxia in normoxic conditions by stabilizing HIF-1 α and blocking its degradation by the ubiquitin/proteosomal pathway [37]. In this study, CoCl₂ was used uniquely as a positive control for expression of HIF-1 α . A2058, A375, and MeWo cells were treated with CoCl₂ at increasing concentrations (0, 50, 100, 200, 400 μ M).

At 24 h post-treatment, results from a western blot showed that HIF-1 α expression increased in a CoCl₂ dose-dependent manner (data not shown). Since HIF-1 α is a nuclear protein, we used Lamin B1 as loading control, which is a major structural protein of the nuclear lamina [39]. Next, melanoma cells were treated with 100 μ M CoCl₂, a concentration that showed no significant toxicity to melanoma cells, as compared to 200 and 400 μ M, and HIF-1 α expression was evaluated. Results show that in A2058 cells HIF-1 α was expressed at 24, 48, and 72 h. This was in contrast with MeWo cells in which HIF-1 α is only expressed up to 48 h (Figure 2a,b) (Figure S1).

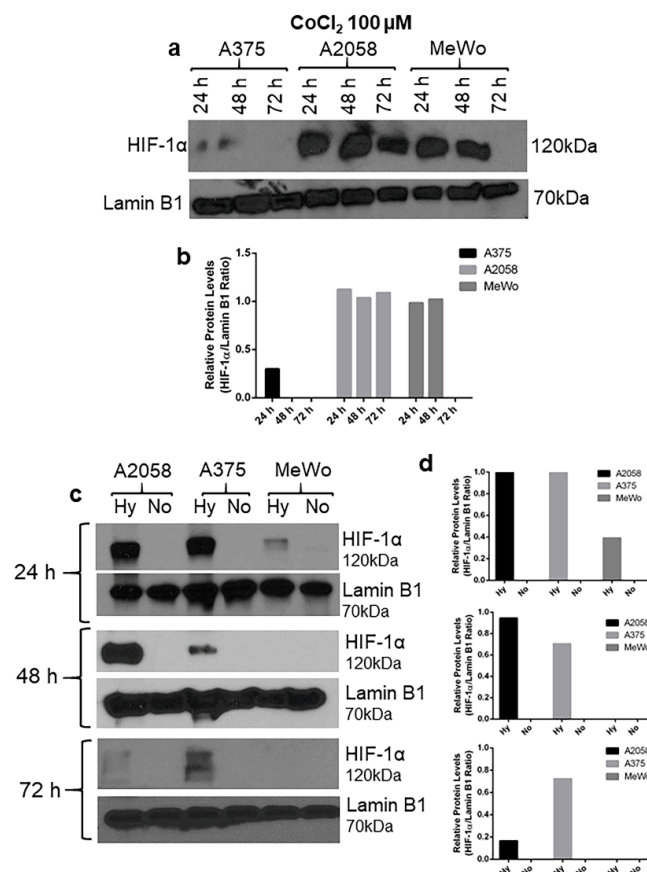


Figure 2. Induction of HIF-1 α expression by cobalt chloride (CoCl₂) or hypoxic conditions: (a) A2058, A375, and MeWo cells were treated with CoCl₂ at a concentration of 100 μ M. Nuclear protein lysates were collected at 24, 48, and 72 h post-treatment. (b) Densitometric quantification of HIF-1 α immunoblots relative to nuclear Lamin B1 protein expression. (c) A2058, A375, and MeWo cells were incubated on hypoxic (Hy) or normoxic (No) conditions. Nuclear protein lysates were collected at 24, 48, and 72 h post-treatment. Expression of HIF-1 α was detected by Western blot; Lamin B1 was used as a loading control. (d) Densitometric quantification of HIF-1 α immunoblots relative to nuclear Lamin B1 protein expression. A representative experiment is shown from three performed.

In order to validate these results, human melanoma cells were cultured in normoxic or hypoxic conditions for 24, 48 and 72 h. Western blot analysis showed HIF-1 α expression in hypoxic conditions and in a time-dependent manner. At 24 h, a strong expression of HIF-1 α in A2058 and A375 cells was observed. This was in contrast with MeWo cells that displayed a weaker signal. At 48 h, HIF-1 α expression decreased in A375 and MeWo cells, and at 72 h, only A375 cells continued to express HIF-1 α . These results confirm that we were able to induce HIF-1 α expression in melanoma cells *in vitro* (Figure 2c,d) (Figures S2 and S3). Moreover, we established an approach to mimic melanoma hypoxia in order to evaluate nisin-mediated *L. lactis* gene expression under hypoxic conditions.

2.3. *Lactococcus Lactis* Expresses Fluorescent Proteins under Hypoxic Conditions

To assess whether *L. lactis* can express fluorescent proteins under hypoxic conditions *in vitro*, *L. lactis*-GFP and *L. lactis*-mCherry strains were co-cultured with MeWo cells in hypoxic and normoxic conditions. Nisin was then added to the culture medium.

At 24 h after co-culture, confocal microscopy revealed that both recombinant strains expressed the transgene in hypoxic and normoxic conditions (Figure 3). This result indicates that nisin inducible promoter can be activated and efficiently express the reporter genes under hypoxic conditions *in vitro*.

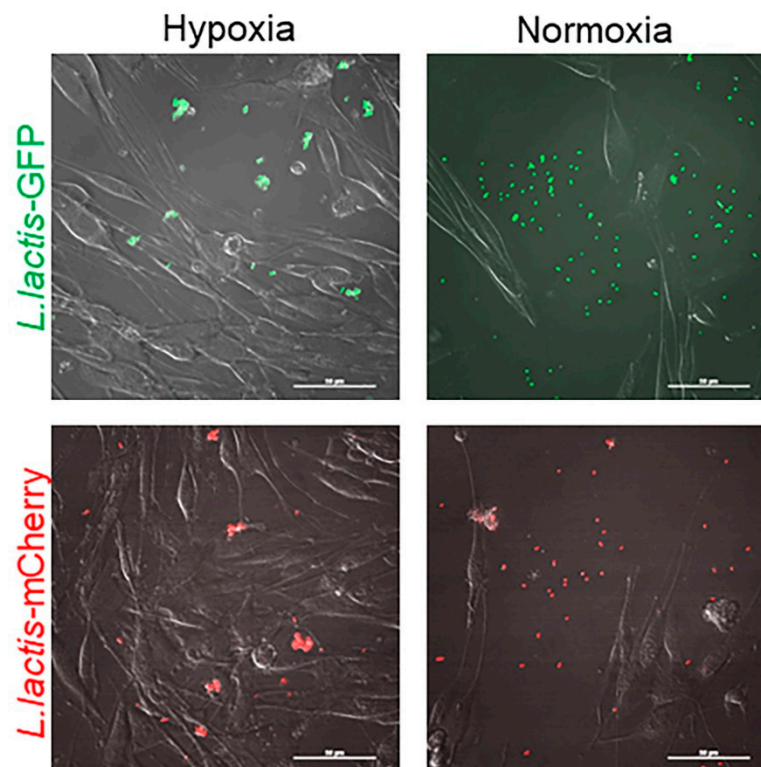


Figure 3. Induction of GFP and mCherry expression under hypoxic conditions: 3×10^5 MeWo cells were cultured in 35 mm glass bottom dishes. *L. lactis*-GFP or *L. lactis*-mCherry were added to the cell culture at a multiplicity of infection (MOI) of 100. Nisin (10 $\mu\text{g}/\text{mL}$) was added to the culture medium. Detection of recombinant *L. lactis* strains was analyzed by confocal microscopy at 24 h post-treatment. Images were taken at 100 \times magnification with the Nikon A1 Confocal System under 587/610 (mCherry) and 488/507 (GFP) nanometers (nm) for excitation and emission visualization, respectively. A representative experiment is shown from three performed. Scale bar = 50 μm .

2.4. Intratumoral Detection of *L. Lactis*-IRFP713 in a Mouse Melanoma Xenograft Model

To determine whether *L. lactis* could be detected in melanoma tumors in vivo, athymic BALB/c mice were implanted with A375 melanoma cells subcutaneously (*s.c.*). When the tumors were palpable ($\sim 100 \text{ mm}^3$), previously induced *L. lactis*-IRFP713 was injected intratumorally (*i.t.*) at increasing colony forming unit (CFU) concentrations and whole-body imaging was performed at different time points. We observed that IRFP713 signal was dose-dependent and detectable in vivo as early as 1 h post-*i.t.* injection and up to 72 h (Figure 4a). To further, evaluate whether *L. lactis* has a predilection for the tumor hypoxic microenvironment, we harvested the melanoma tumors and were subjected to immunohistochemistry (IHC) for HIF-1 α and Gram staining.

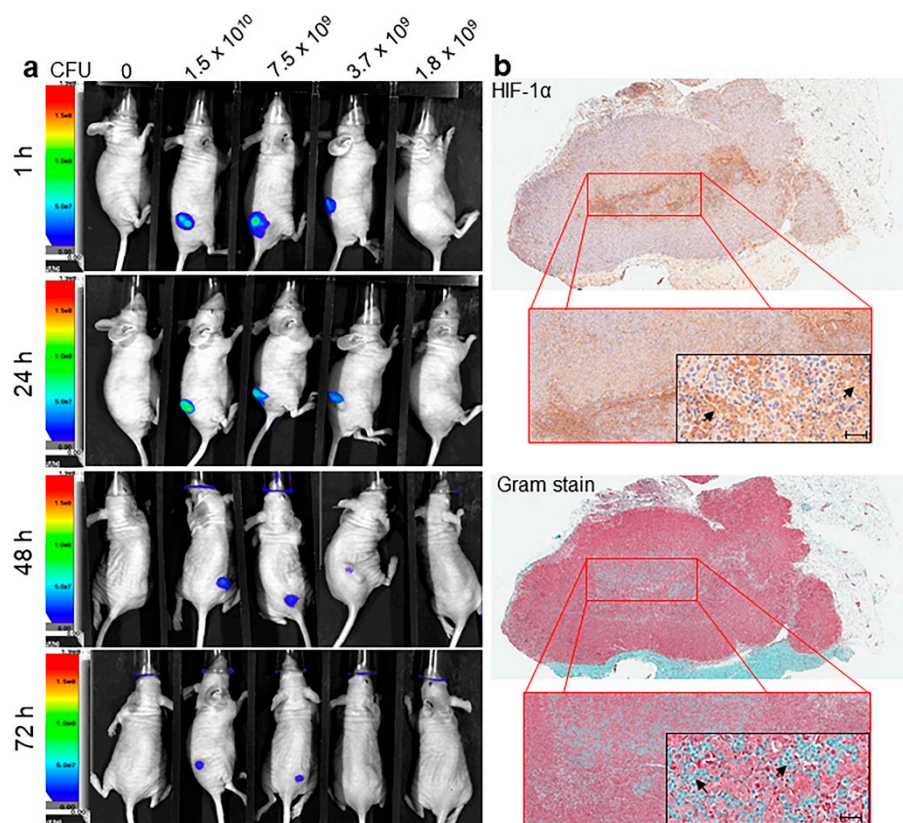


Figure 4. Detection of *L. lactis*-IRFP713 via advance molecular imaging and co-localization of *L. lactis* with HIF-1 α in the core of the melanoma tumor. (a) Representative time-course imaging of tumor bearing mice administered with IRFP713-expressing *L. lactis*. BALB/c mice were administered 1×10^6 A375 cells in the right flank by *s.c.* injection. Ten days after tumor inoculation, mice were administered IRFP713-expressing *L. lactis* at increasing concentrations (0 , 1.8×10^9 , 3.7×10^9 , 7.5×10^9 and 1.5×10^{10} CFU) by *i.t.* injection. Whole body imaging was performed at 1, 24, 48 and 72 h with the AMI-1000-X instrument under 690/713 nanometers (nm) excitation and emission visualization, respectively; (b) Representative IHC staining of HIF-1 α and Gram staining of A375 tumor sections from BALB/c mice injected *i.t.* with *L. lactis*-IRFP713 at a magnification of $\times 100$ and $\times 400$. Arrows indicate gram-positive bacteria cells and cells positive for HIF-1 α , respectively. Whole tissue slides were scanned with Leica Aperio ImageScope with $40\times$ magnification. Scale bar = $50 \mu\text{m}$.

The IHC staining revealed a strong positive-HIF-1 α staining in the core of the tumor (Figure 4B). The Gram staining revealed the presence of gram-positive bacteria predominantly in the center of the necrotic areas of the tumor along with HIF-1 α staining. In order to demonstrate that HIF-1 α expression was not due to necrosis induced by the injection trauma, we performed an IHC staining for HIF-1 α in A375 tumors that were not injected, results show HIF-1 α expression in the non-injected tumor (Figure S4).

To further validate that the iRFP713 signal from Figure 4a declined due to bacteria clearance, A375 tumors previously injected with 1.5×10^{10} CFU *L. lactis*-IRFP713 or PBS 1x were collected and homogenized at 24, 48, and 72 h post-injection. The homogenate was then serially diluted and plated in GM17 agar plates. At 24 h post-incubation CFU were determined. Results show that the number of CFU decreased in a time-dependent manner, from an average 6.83×10^5 CFU/mL at 24 h, to 1.11×10^5 CFU/mL at 48 h, and at 72 h bacterial colonies were no longer detected (Figure S5). Altogether, these results show both: (i) that *L. lactis* efficiently expresses IRFP713 *in vivo* and was detectable up to 72 h after *L. lactis* injection and ii) that *L. lactis* can efficiently express the transgene into the solid tumor for at least 72 h.

2.5. *L. Lactis* Expressing β -Galactosidase (*L. Lactis*- β -Gal) as a Promising Contrast Agent for Multispectral Optoacoustic Tomography (MSOT)

The expression of β -galactosidase is regulated by the stress-inducible controlled expression system (SICE), which is based on the use of the promoter from *L. lactis* *groESL* operon, which neither requires the presence of regulatory genes, nor the induction of the cultures before use (i.e., administration of the strain into the host).

The advantage of this system is that *L. lactis* will be able to deliver proteins of health interest *in situ* without the need of previous *in vitro* induction (e.g., nisin addition in NICE system) [28]. However, for a better detection of β -galactosidase in *in vitro* tests, the SICE system was induced by incubation of *L. lactis* cultures at 37 °C for 1 h and the substrate X-gal was added to the culture. Three hours later, the bacterial suspension was centrifuged into a blue pellet as shown in Figure 5a.

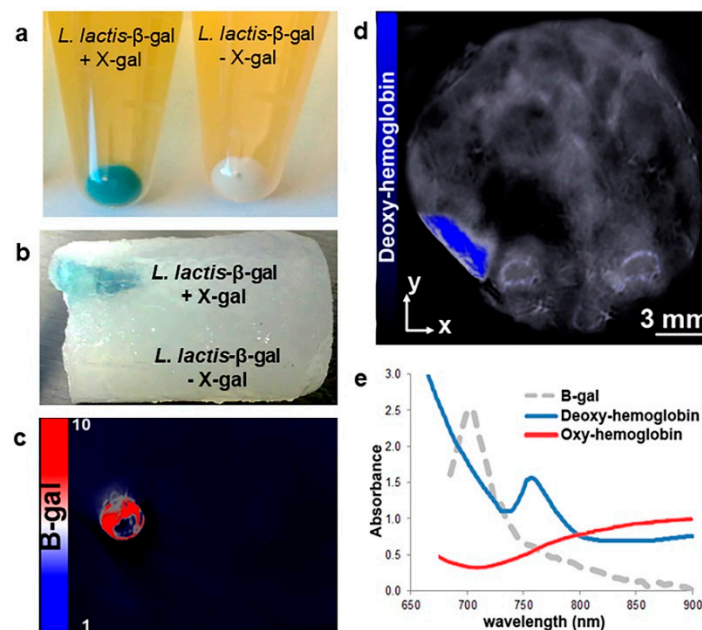


Figure 5. Characterization of *L. lactis*- β -gal as a contrast agent for *in vivo* MSOT imaging. (a) *L. lactis*- β -gal strain was cultured at 30 °C without agitation in GM17 (OD600 nm = 0.6–0.8). Cultures were incubated for 1 h at 37 °C, and then X-gal was either added or not added to the cultures. Three hours later, a blue cell pellet was obtained by centrifugation. A pellet of cells from each culture is shown. (b) Cylindrical phantoms with a diameter of 2 cm were prepared using a gel made from distilled water, agar, and an intralipid 20% emulsion (to simulate light diffusion *in vivo*). (c) Phantoms containing *L. lactis*- β -gal with (left) or without (right) X-gal were imaged by MSOT using a transversal plane. (d) The orthogonal image shows tissue hypoxia of A375 tumors as corresponding to deoxygenated hemoglobin (blue bar). (e) The analysis of *L. lactis*- β -gal + X-gal and deoxy-hemoglobin spectra does not overlap, which suggests that both could be used simultaneously for multispectral unmixing of MSOT images.

To determine whether *L. lactis*- β -gal could be detected by MSOT, a phantom experiment was performed. Cylindrical phantoms (Figure 5b) with a diameter of 2 cm were prepared using a gel composed of distilled water, agar, and an intralipid 20% emulsion (to simulate light diffusion in vivo). The cell pellets from Figure 5a were resuspended in 100 μ L of phosphate buffered saline (PBS) 1 \times , and a 20 μ L of the suspension was loaded into the phantom, which was then scanned by MSOT. After image reconstruction, according to our previous report [28], the image analysis revealed a clear and strong signal where *L. lactis*- β -gal + X-gal was loaded, whereas no signal was detected where only *L. lactis*- β -gal was loaded (Figure 5c). This result indicates that *L. lactis* expressing β -galactosidase has a great potential as a contrast agent for MSOT imaging.

2.6. The Spectrum of *L. Lactis*- β -Gal + X-Gal does not Overlap with Oxy- and Deoxy-Hemoglobin

A subcutaneous A375 melanoma tumor was implanted and imaged using MSOT. Whole body imaging was performed using transversal slices with a 0.2-mm step from the liver to the kidney (38–56 mm) at wavelengths of 680, 710, 730, 740, 760, 770, 780, 800, 950, 900 nm for each position. Twenty-five averages per wavelength with an acquisition time of 10 microseconds (ms) per frame minimized the influence of animal movement in the images. Figure 5d shows a melanoma tumor with deoxy-hemoglobin, which indicates large areas of tissue hypoxia. The absorbance spectrums of *L. lactis*- β -gal + X-gal, oxy-, or deoxy-hemoglobin were analyzed (Figure 5e), revealing no overlap. *L. lactis*- β -gal + X-gal can be distinguished from oxy- or deoxy-hemoglobin spectrums to allow for simultaneous detection of β -galactosidase and independent identification of tumor oxygenation. Therefore, we consider feasible the detection and tracking of *L. lactis* in melanoma tumors in vivo using MSOT imaging.

2.7. Tracking of *L. Lactis*- β -Gal in Melanoma Tumors In Vivo Using MSOT

In order to determine the feasibility to track *L. lactis* in vivo and its potential to establish in tumor hypoxic niches, we used MSOT. To facilitate the use of MSOT to detect *L. lactis* in vivo, we used *L. lactis* expressing IRFP713 (*L. lactis*-IRFP713) or β -galactosidase (*L. lactis*- β -gal). BALB/c mice bearing A375 tumors were imaged at 1, 24, and 48 h post-*L. lactis* i.v. injection. In this assay, A375 tumors were generated in the lower abdominal region, as previous studies have shown this to be the ideal region for MSOT imaging [40]. At 24 h, the reconstruction of MSOT images revealed co-localization of *L. lactis*- β -gal + X-gal with deoxy-hemoglobin, indicating areas of tumor hypoxia (Figure 6a).

Since *L. lactis*-IRFP713 signals were too weak to be identified with MSOT (data not shown), we used near infrared imaging (NIR) to detect *L. lactis*-IRFP713 accumulation into melanoma tumor following i.v. injection. We observed that mice injected with *L. lactis*-IRFP713 had identifiable signals, whereas the blue color- β -gal did not demonstrate fluorescent signals (Figure 6b). In the group of mice injected with *L. lactis*-IRFP713, we observed background signal underneath the tumor, which we have observed in previous studies using the same NIR settings [41]. These results suggest that *L. lactis* had the ability to find and establish preferentially within the tumor hypoxia niches. These findings suggest that *L. lactis* represents an attractive agent to target and deliver therapeutic molecules into the hypoxic tumor microenvironment.

3. Discussion

In this report, we describe the evaluation of lactic acid bacterium *L. lactis* targeting the tumor hypoxic microenvironment. We show that *L. lactis* is able to express reporter genes IRFP713 and β -galactosidase in vivo and in hypoxic conditions *in vitro*, *L. lactis* remained in the tumor tissue for up to 72 h and, most importantly, is non-pathogenic to humans and animals. Bacteria-based cancer gene therapy represents an alternative approach that has gained significant attention, mainly due to the unique tumor colonizing ability of bacteria [42,43]. The desirable characteristics for using facultative anaerobic bacteria as a cancer-targeting gene delivery vehicle are that: (1) they are self-propelled and penetrate into poorly vascularized tissue regions; (2) they have the ability to target hypoxic regions

to improve tumor specificity; and (3) they are amenable to genetic manipulation [22]. Although the initial focus of utilizing bacteria as an anti-cancer gene delivery vehicle has centered upon *Salmonella*, *Listeria*, and *Clostridium* strains [22,44,45], the inherent risk of reversion to a virulent strain via gene transfer or mutation substantially limits their application in a clinical setting. Moreover, bacteria-based cancer gene therapy can overcome a common limitation with other modalities of gene and molecular therapies such as the failure to deliver therapeutic molecules in the majority of the cells within a three-dimensional solid tumor mass.

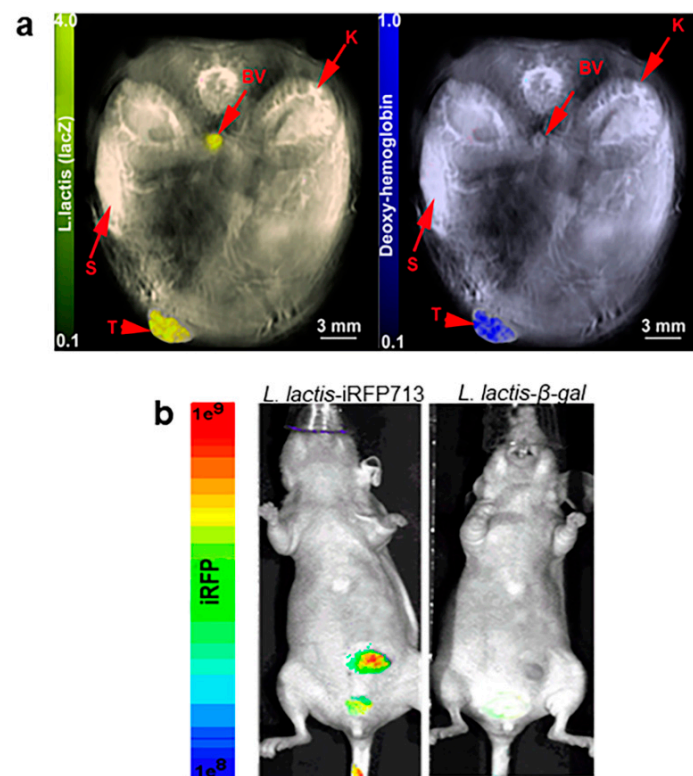


Figure 6. Feasibility of *L. lactis-β-gal* to establish within areas of tumor hypoxia using MSOT or near infrared imaging: (a) *L. lactis-β-gal* (1.5×10^9 CFU/50 μ L) was i.v. injected into mice and detected within tumor. Mice were anesthetized using 0.1 L O₂ and 0.9 L medical air with 1.5% isoflurane for imaging following 24 h post-injection using MSOT. Mouse images were reconstructed and multispectrally processed using standard techniques. (Left) *L. lactis-β-gal* was demonstrated within the hypoxic regions of the tumor as depicted by deoxy-hemoglobin (Right). Organs are indicated using arrows: blood vessel (BV); kidney (K); spleen (S); tumor (T). (b) Feasibility of detection of *L. lactis-IRFP713* using NIR fluorescence imaging. Mice i.v. injected with *L. lactis-IRFP713* had identifiable signals (left), whereas the blue color-LacZ does not demonstrate fluorescent signals (right). A representative MSOT or NIR images at 24 h post-intravenous injection of *L. lactis* is shown from three performed.

Tumor hypoxia is a component of the tumor microenvironment that can influence most of the stages of metastasis. Approximately 50–60% of locally advanced solid tumors, including melanoma, show areas of hypoxia/anoxia that contain some of the most malignant cells. Metastasis is a complex multi-step process in which tumor cells have to acquire an invasive/migratory phenotype, enter the blood and lymphatic flow, survive anoikis and the immune response, and once localized to a distant site, survive in a likely hostile environment and proliferate [46,47]. In our study, we observed that metastatic lymph-node derived MeWo cells adapted more efficiently to the harsh hypoxic environment, which resulted in an increased survival rate, when compared to skin-derived non-metastatic A2058 and A375 cells.

Presently, the two most common strategies to detect bacterial accumulation are either bioluminescence or fluorescence imaging. While both of these mechanisms are successful in detecting bacterial accumulation within subcutaneous tumors or at surface depths, light-based imaging systems suffer from photon scattering, 2D presentation, and signal attenuation at depths >5 mm, which preclude clinical application [48]. MSOT is a newly emerging technology that, overcomes the limitations abovementioned. Recently, MSOT has been used to image sentinel lymph nodes (SLNs) in patients with melanoma to determine metastatic status [49], MSOT combined with the near-infrared fluorophore indocyanine green reliably visualized SLNs in vivo in 20 patients, up to 5 cm penetration, and with 100% concordance with ^{99m}Tc-madked SLN lymphoscintigraphy. Recently, high intensity focused ultrasound (HIFU) irradiation was used to detect targeting of solid tumors with anaerobic bacterium *Bifidobacterium longum* (*B. longum*). In this study, *B. longum* served as a vehicle to conjugate with poly (lactic-co-glycolic acid) (PLGA) nanoparticles (PLGA NPs). Results showed that after *i.v.* administration, *B. longum* was able to reach MDA-MB-231 tumors, and was retained for up to 168 h, one limitation of this strategy is that the PFH/PLGA NPs have to be fabricated via a double emulsion method and then connected to the bacterial surface of *B. longum* [50].

In our study, we used BALB/c mice to evaluate tumor colonization by *L. lactis* using MSOT. BALB/c mice are an immunodeficient athymic strain that have a strongly reduced number of T cells. In this group of mice, intratumoral and intravenous administration of *L. lactis* resulted in tumor colonization up until 72 h and 24 h respectively. Further studies in wild-type mice will be needed to determine whether *L. lactis* is able to persist in the tumor tissue without being eliminated by the animal immune system. In this study, we detected the hypoxia marker HIF-1 α predominantly in the core of the tumor, however this could also be an effect of necrosis induced by the injection trauma, we consider that this could represent a limitation in this study. Moreover, while a subcutaneous melanoma model is useful as proof-of-concept, it will be important to evaluate whether *L. lactis* can target and accumulate in a melanoma liver metastasis model. Melanoma metastasis causes the vast majority of morbidity and mortality associated with this disease, and the presence of metastasis to visceral sites predicts poor outcome in melanoma [51,52]. Finally, the strategy proposed in this study has a therapeutic potential, by engineering *L. lactis* to express and deliver molecules of health interest directly in the hypoxic microenvironment of solid tumors. *L. lactis* has been used as a vehicle to deliver therapeutic molecules into the human body, including cytokines/ligands (e.g., IL-10, IL-22, RANKL, TGF- β 1) [53–55], tumor-associated antigens (TAAs) (e.g., HPV-16 E7, TRP-2) [56,57], antioxidant enzymes [58], and protease inhibitors [59].

In summary, we showed that *L. lactis* is a promising, tumor-targeting agent to deliver therapeutic molecules into the tumor hypoxic microenvironment. Furthermore, to our knowledge we are the first to describe the application of *L. lactis* as a contrast agent for MSOT. *L. lactis* expressing β -galactosidase was able to be detected in melanoma tumors in vivo and in real time, moreover, the β -gal signal did not overlap with oxygenation markers, oxy- and deoxy- hemoglobin which can allow simultaneous detection of β -galactosidase and independent identification of tumor oxygenation.

4. Materials and Methods

4.1. Cell lines and Culture Conditions

Skin derived human melanoma cell lines A2058 (Cat# CRL-11147), A375 (Cat# CRL-1619) and metastatic lymph-node derived melanoma cell line MeWo (Cat# HTB-65) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A2058 and A375 cells were grown in DMEM medium whereas MeWo cells were grown in MEM medium, all mediums were supplemented with 10% FBS. All cells were maintained at 37 °C in a 5% CO₂ humidified incubator. All cell culture reagents were obtained from Corning Cellgro (Manassas, VA, USA).

4.2. Bacterial Strains and Growth Conditions

The *L. lactis*-GFP and *L. lactis*-mCherry strains used in this study were previously reported by our group [26], both were grown in M17 medium (Becton Dickinson Co., Franklin Lakes, NJ, USA) supplemented with 1% (w/v) glucose (GM17) and 10 µg/mL chloramphenicol at 30 °C without agitation and aeration. The plasmid pNZ-IRFP713 was kindly provided by Aleš Berlec (Jožef Stefan Institute, Ljubljana, Slovenia) [60]. Electrocompetent *L. lactis* NZ9000 were transformed with pNZ-IRFP713 according to Holo [61], by electroporation, using Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA). For selecting recombinant clones, *L. lactis* was plated in GM17 agar containing 10 µg/mL chloramphenicol.

4.3. Construction of a Vector for Stress-Inducible β -Galactosidase Production in *L. Lactis*

For the construction of a plasmid allowing controlled-cytoplasmic β -galactosidase production in *L. lactis* under the SICE system [28], *lacLM* genes were obtained from P_{NICE}:SEC:LacLM plasmid (unpublished data) after digestion with *NsiI/SpeI* and cloned into purified vector isolated from *NsiI/SpeI*-cut P_{SICE}:CYT:TagD vector (unpublished data). The resulting plasmid P_{SICE}:CYT:LacLM was established by electroporation into *L. lactis* MG1363 strain. This vector allows a cytoplasmic β -galactosidase production under the transcriptional control of a *L. lactis*-stress-inducible promoter: *groESL*. The β -galactosidase expression was confirmed as follows: *L. lactis*- β -gal strain was cultured at 30 °C without agitation in GM17 media (OD_{600 nm} = 0.6–0.8). Cultures then were incubated for 1 h at 37 °C, and the substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, 200 µg/mL) was either added or not added to the cultures. Three hours later a blue cell pellet was obtained by centrifugation.

4.4. Induction of Reporter Gene Expression and Visualization of GFP, mCherry and IRFP713

Cultures of recombinant strains of *L. lactis* were incubated until an optical density at 600 nm (OD₆₀₀) of 0.6–0.8 was reached. We added 10 µg/mL nisin (Cat# N5764, Sigma-Aldrich, St. Louis, MO, USA) to the cultures of *L. lactis*-GFP and *L. lactis*-mCherry. Two hours after induction of gene expression with nisin, 1 mL of culture was centrifuged at 23,000× *g* for 10 sec and re-suspended in 20 µL phosphate-buffered saline (PBS) 1×. To visualize the expression of the reporter genes, first 10 µL of bacteria suspension was placed on slides, and then a Leica DM1000 fluorescence microscope with an N2.1 filter was used for mCherry at 587 and 610 nm for excitation and emission, respectively, and an I3 filter for GFP at 488 and 507 nm for excitation and emission, respectively.

For IRFP713 visualization, after reaching an OD₆₀₀ of 0.6–0.8, *L. lactis* expressing IRFP713 was induced with 10 µg/mL nisin for 2 h. Biliverdin-HCl (Cat #30891, Sigma-Aldrich, Saint Louis, MO, USA) was added in bacterial growth medium at a concentration of 15.5 µg/mL when culturing this strain for both in vitro and in vivo experiments. One mL of culture was centrifuged at 23,000× *g* for 10 s. A microcentrifuge tube containing the pellet was observed using an Advanced Molecular Imager 1000-X (AMI1000-X; Spectral Imaging Instruments, Tucson, AZ, USA) instrument under 690/713 nanometers (nm) excitation and emission visualization, respectively.

4.5. Induction and Testing of Hypoxia

To establish hypoxic conditions, melanoma cells were placed in a hermetically sealed, humidified modular incubator chamber (Cat# MIC-101, Billups-Rothenberg, Inc., San Diego, CA, USA). Hypoxic conditions were obtained by flushing the incubator chamber with a gas mixture of 5% CO₂, 94% N₂ and 1% O₂ for 20 min at 2 psi. The hypoxic chamber was then placed in a conventional incubator for 24, 48 or 72 h. Humidity within the chamber was maintained during the experiments by placing a plastic petri dish containing 10 mL of sterile water [38]. Cells from hypoxic and normoxic cultures were processed immediately after incubation. Cells incubated on normoxic conditions were used as a

control. CoCl_2 (Cat #C8661, Sigma-Aldrich), a chemical hypoxia-inducing agent, was used in vitro to mimic the effects produced in the modular incubator chamber.

4.6. Western Blot Analysis

Nuclear proteins were extracted from hypoxic and normoxic cells using a NE-PER nuclear and cytoplasmic extraction kit (Cat# 78833, Thermo-Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Equal amounts of nuclear protein were electrophoresed in 10% SDS-polyacrylamide gels and transferred to hybond-PVDF membranes (Cat# 23225, GE Healthcare Life Sciences, Pittsburgh, PA, USA). The primary antibodies used in this study were: rabbit anti-Lamin B1 (Cat# ab133741, Abcam, Cambridge, MA, USA) and rabbit anti-HIF-1 α (D2U3T) (Cat# 14179, Cell Signaling Technology, Danvers, MA, USA). Next, the membranes were incubated with anti-rabbit Ig, peroxidase-linked, species-specific whole antibody (Cat# 31460, Thermo-Fisher Scientific). ECL reagents were used to detect the signals according to the manufacturer's instructions (Cat# RPN3244, GE Healthcare Life Sciences, Pittsburgh, PA, USA). All films were scanned with an Epson Expression 1680 optical scanner (Epson America Inc., Long Beach, CA, USA). Densitometry analysis of bands was performed using an open-source, public domain software package (ImageJ v1.47).

4.7. MTT Assay

Inhibition of cell viability was assessed 24, 48 and 72 h after adding CoCl_2 or incubating in normoxic or hypoxic conditions by measuring the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to formazan according to the manufacturer's instructions (Cat #M2128, Sigma-Aldrich) and as described by our group [62]. The supernatant from each plate was collected for measurement of absorbance at a wavelength of 570 nm. The results were expressed as the percentage of live cells. For negative control groups, we used cell line-specific media alone without CoCl_2 or cells incubated in normoxic conditions.

4.8. Co-Culture of Recombinant *L. Lactis* Strains with Human Melanoma Cells

Metastatic lymph-node derived human melanoma cell line MeWo was plated in a 24-well plate at a density of 1×10^6 cells. *L. lactis*-GFP or *L. lactis*-mCherry were grown as described previously. These strains were pelleted and washed twice in PBS 1x, and then added to the cell cultures at a multiplicity of infection (MOI) of approximately 100 bacteria per eukaryotic cell. Nisin (Cat# N5764, Sigma-Aldrich) was added to the co-culture at a concentration of 10 $\mu\text{g}/\text{mL}$ and the cell plates were incubated in normoxic or hypoxic conditions for 24 h. Transgene expression was evaluated using a Nikon A1R confocal microscope (Nikon Inc., Melville, NY, USA) at 100 \times magnification under 587/610 (mCherry) and 488/507 (GFP) nanometers (nm) for excitation and emission visualization, respectively.

4.9. Signal Assessment Ex Vivo in Tissue Phantoms

To determine the potential of *L. lactis*- β -gal as a contrast agent for MSOT imaging, *L. lactis*- β -gal was added to tissue phantoms designed to simulate optical properties of murine tissue. The tissue phantom was constructed according to the following procedure: fixed cylindrical phantoms of 2 cm in diameter were prepared using a gel made from distilled water containing agar (Sigma Aldrich) for jellification (1.3% w/w) and an intralipid 20% emulsion (Sigma Aldrich) for light diffusion (6% v/v), resulting in a gel presenting a reduced scattering coefficient of $\mu'_s \approx 10 \text{ cm}^{-1}$. *L. lactis*- β -gal (+) X-gal or *L. lactis*- β -gal (−) X-gal samples were inserted into cylindrical inclusions approximately 3 mm in diameter. The gel used to construct the tissue phantom was used to seal the samples in the appropriate well prior to MSOT imaging. MSOT imaging of the phantoms was obtained as described previously [28]. Data in MSOT arbitrary units (a.u.) were statistically compared using ANOVA.

4.10. Mice Studies

Female (6- to 8-wks-old) BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in a barrier animal facility at the University of Louisville in accordance with NIH guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 8023, Revised 1978), all procedures were analyzed and approved by the University of Louisville IACUC (protocol no. 17003).

Groups of five athymic BALB/c were implanted with $1 \times 10^6/100 \mu\text{L}$ A375 melanoma cells subcutaneously (s.c.) in the right flank. Ten days after tumor inoculation, mice were injected intratumorally (i.t.) with nisin-induced *L. lactis*-IRFP713 at a colony forming unit (CFU) concentration of 1.5×10^{10} , 7.5×10^9 , 3.75×10^9 or 1.8×10^9 . Whole body imaging was performed at 1, 24, 48 and 72 h using an AMI-1000-X instrument under 690/713 nanometers (nm) excitation and emission visualization, respectively.

4.11. Immunohistochemistry and Gram Staining of A375 Tumors

To visualize cellular morphology, A375 tumor sections were stained with hematoxylin and eosin (H&E). Gram staining of A375 tumor specimens was performed using a Gram stain kit (#AR17592-2, Agilent Technologies, Santa Clara, CA, USA) following the standard protocol for paraffin specimens and according to the manufacturer's instructions. For immunohistochemistry, tumors were excised, fixed in 10% formalin, embedded in paraffin blocks, and processed for histological analysis. Rabbit anti-HIF-1 α (D2U3T) (Cat# 14179, Cell Signaling Technology, Danvers, MA, USA) was used at a 1:500 concentration to detect expression of HIF-1 α . The slides were then washed with PBS 1 \times , incubated with the standard ultra-sensitive ABC peroxidase staining kit (Pierce, Rockford, IL, USA), and detected with diaminobenzidine (DAB) tetrahydrochloride solution containing 0.0006% H₂O₂. Hematoxylin was used as a counterstain. Whole tissue slides were scanned with Leica Aperio ImageScope with 40 \times magnification (Leica Biosystems, Buffalo Grove, IL, USA).

4.12. In Vivo Bacterial Colonization

For determination of tumor bacterial count, BALB/c mice were implanted with $1 \times 10^6/100 \mu\text{L}$ A375 melanoma cells subcutaneously (s.c.) in the right flank. Ten days after tumor inoculation, mice were injected intratumorally (i.t.) with PBS 1 \times or *L. lactis*-IRFP713 at a CFU concentration of 1.5×10^{10} . At 24, 48, or 72 h following *L. lactis*-IRFP713 administration, mice were euthanized, and their tumors were aseptically excised, dissected, homogenized with a tissue tearor (BioSpec Products, Bartlesville, OK, USA) in 1 mL of PBS. The homogenate was plated in serial dilutions on GM17 agar containing 10 $\mu\text{g/mL}$ chloramphenicol and incubated at 30 $^\circ\text{C}$ for 24 h. The surface viable count technique of Miles and Misra was employed for counting of the number of CFUs [63].

4.13. In Vivo Imaging and Reconstruction

Athymic BALB/c mice were implanted with A375 melanoma cells subcutaneously. When the tumors were palpable ($\sim 100 \text{ mm}^3$), mice were separated in two groups. Group 1 ($n = 5$) mice were injected i.v. with *L. lactis*-IRFP713 or Group 2 ($n = 5$) *L. lactis*- β -gal at a dose of 1.5×10^9 CFU/50 μL . MSOT imaging was performed as described previously by us [64]. Mice were anesthetized with 1.6% isoflurane and prepped for imaging with a combination of Nair cream with aloe (Church and Dwight Co., Princeton, NJ, USA) and shaving. The mice were imaged using MSOT system InVision TF 256 (iThera Medical, Munich, Germany). Serial slice images were taken in 0.2 mm steps between the diaphragm to the bottom of the kidneys (40–55 mm) at wavelengths of 680, 710, 730, 740, 760, 770, 780, 800, 850, and 900 nm using 25 averages per wavelength with acquisition time of 10 μs per frame in order to minimize the influence of animal movement. Images were taken at 4, 8, 16, and 24 h post-injection to track *L. lactis* uptake and accumulation into the tumor. Excitation of *L. lactis*- β -gal (+) X-gal was conducted using a tunable parametric oscillator pumped by an Nd:YAG laser. The melanoma tumor

was identified by a live-feed screen preview multispectral signal. Video-rate acquisition produced an imaging clip compiled from single-slice acquisition in less than 1 ms, resulting in an image data rate of 10 frames per second. Image reconstruction was conducted using backprojection at a resolution of 75 μm . Multispectral processing was conducted using linear regression (ViewMSOT 3.5)

4.14. Statistical Analysis

The results of the in vitro assays were analyzed by unpaired Student's *t* test using a one-way ordinary parametric analysis of variance. The statistical analysis was performed using Prism software (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ indicated a statistically significant difference.

5. Conclusions

This study demonstrates that the LAB *L. lactis* is able to express fluorescent reporter proteins GFP and mCherry in vitro under hypoxic conditions and is able to target, find and establish within tumor hypoxic niches in vivo. We show that intravenously administered *L. lactis* expressing IRFP713 or β -galactosidase had the ability to find and establish preferentially within the tumor hypoxia niches.

In conclusion, our data suggests that *L. lactis* represents an alternative strategy to target and deliver therapeutic molecules into the tumor hypoxic microenvironment.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/2/438/s1>, Figure S1: Representative whole blot. (a) A2058, A375, and MeWo cells were treated with CoCl_2 at a concentration of 100 μM . Nuclear protein lysates were collected at 24, 48, and 72 h post-treatment. Expression of HIF-1 α was detected by Western blot; (b) Lamin B1 was used as a loading control, Figure S2: Representative whole blot. (a) A2058, A375, and MeWo cells were incubated on hypoxic (Hy) or normoxic (No) conditions. Nuclear protein lysates were collected at 24 h post-treatment. Expression of HIF-1 α was detected by Western blot; (b) Lamin B1 was used as a loading control, Figure S3; Representative whole blot. (a) A2058, A375, and MeWo cells were incubated on hypoxic (Hy) or normoxic (No) conditions. Nuclear protein lysates were collected at 48 h post-treatment. Expression of HIF-1 α was detected by Western blot; Lamin B1 was used as a loading control, (b) Cells were treated as mentioned above, nuclear protein lysates were collected at 72 h post-treatment. Expression of HIF-1 α was detected by Western blot; Lamin B1 was used as a loading control, Figure S4. Representative Hematoxylin and Eosin (H&E) staining and IHC staining of HIF-1 α A375 tumor sections from non-injected BALB/c mice at a magnification of x100 and x400. Arrows indicate cells positive for HIF-1 α . Whole tissue slides were scanned with Leica Aperio ImageScope with 40x magnification, Scale bar = 50 μm , Figure S5: A375 tumors from BALB/c mice were homogenized at 24, 48 and 72 h following *L. lactis*-IRFP713 administration or PBS 1x. The homogenate was plated in serial dilutions on GM17 agar containing 10 $\mu\text{g}/\text{mL}$ chloramphenicol.

Author Contributions: R.G.-M. and J.G.G.-G. wrote the paper. R.G.-M., M.T.M., L.R.M. and J.G.G.-G. performed the experiments. A.A. and L.G.B.-H. constructed and characterized *L. lactis* expressing β -galactosidase. R.G.-M., B.E.R., J.E.G.-C., L.R.M., K.M.M., M.T.M. and J.G.G.-G. conceived and designed the experiments, analyzed the data and edited the manuscript. R.G.-M., L.R.M., K.M.M., L.G.B.-H. and J.G.G.-G. revised the manuscript. All authors attest they meet the ICMJE criteria for authorship. All authors have read and agree to the published version of the manuscript.

Funding: This work was supported by the NIH/NCI award number R21CA210202 and Burroughs Wellcome Fund award number 1018775 (J.G.G.-G.). M.T.M. is supported by NIH grant number 5T32HL134664-3.

Acknowledgments: We thank Margaret Abby for editing. We thank Aleš Berlec (Jožef Stefan Institute, Ljubljana, Slovenia) for kindly providing us plasmid pNZ-IRFP713.

Conflicts of Interest: The authors declare no conflict of interest.

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