Oxygen Tolerance Domestication of *Blautia* sp. AUH-JLD56 Enables Efficient Aerobic Bioconversion of Arctigenin to 3'-Demethylarctigenin

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Article

Oxygen Tolerance Domestication of *Blautia* sp. AUH-JLD56 Enables Efficient Aerobic Bioconversion of Arctigenin to 3′-Demethylarctigenin

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Simple Summary

The original bacterial strain *Blautia* sp. AUH-JLD56, capable of anaerobically converting arctigenin to 3′-demethylarctigenin (3′-DMAG), is a strict anaerobe isolated from human feces. However, long-term maintenance under strict anaerobic conditions is expensive and inconvenient. To overcome this problem, we carried out oxygen tolerance domestication of the strictly anaerobic wild-type strain and successfully obtained an oxygen-tolerant mutant, which we named *Blautia* sp. Aeroto-AUH-df6. This mutant grows and proliferates normally in the presence of atmospheric oxygen and efficiently converts arctigenin under aerobic conditions, achieving a maximum concentration of 8.2 mM, which is 2.3 times that (3.6 mM) achieved by the wild-type strain under anaerobic conditions. The findings of this study have practical importance for intestinal anaerobic microorganisms. Given their oxygen-sensitive nature, the oxygen tolerance domestication approach described here can be applied to select and obtain mutant strains capable of normal growth and efficient conversion under conventional aerobic conditions.

Abstract

Anaerobic bacteria are the dominant group in the animal intestinal microbiota, and most strains cannot grow or proliferate normally upon exposure to air. *Blautia* sp. AUH-JLD56 (KF374935) is a strictly anaerobic strain previously isolated by our research group from human feces. Under anaerobic conditions, this strain converts arctigenin to 3′-demethylarctigenin (3′-DMAG), reaching a maximum conversion concentration of 3.6 mM. To improve the oxygen tolerance of this wild-type strain, we performed long-term oxygen tolerance domestication and successfully obtained an oxygen-tolerant mutant. Phenotypic analysis showed that the growth of the oxygen-tolerant mutant under aerobic conditions ($OD_{600\ nm}$ = 2.37) was slightly lower than that of the wild-type under strictly anaerobic conditions ($OD_{600\ nm}$ = 2.82). Compared with the wild-type, the mutant exhibited an accelerated aerobic growth rate and enabled stable conversion of arctigenin. Notably, under aerobic conditions, the mutant achieved a maximum conversion concentration of 8.2 mM, which is significantly higher than the 3.6 mM obtained with the wild-type under anaerobic conditions. This study realizes, for the first time, efficient aerobic bioconversion of arctigenin to 3′-DMAG using an oxygen-tolerant derivative of a strict anaerobe, thereby overcoming the oxygen-dependent limitation of such strains. Our approach provides a new strategy and technical reference for the oxygen tolerance domestication and industrial application of other intestinal strict anaerobes with specific enzymatic functions.

Keywords: oxygen tolerance domestication; microbial bioconversion; arctigenin; 3′-demethylarctigenin (3′-DMAG)

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1. Introduction

Microbial bioconversion technology utilizes one or more functional enzymes synthesized by microorganisms to catalyze the transformation of substrates at specific sites. It has already achieved industrial application in areas such as steroid hormone synthesis [1–4]. In recent years, this technology has been increasingly applied in the research and development of traditional Chinese medicine. *Arctii Fructus*, the dried ripe fruit of *Arctium lappa* L., is a commonly used traditional Chinese medicine known for its pharmacological effects, including clearing heat, reducing swelling, detoxifying, promoting eruption, and relieving sore throat. Arctiin and arctigenin are the most abundant lignan active components in *Arctii Fructus*. Existing studies have confirmed that arctigenin possesses various pharmacological activities, including anti-inflammatory [5], antitumor [6], renoprotective [7], antifibrotic [8], and neuroprotective [9] effects.

In clinical practice, traditional Chinese medicines are mostly administered orally. After oral administration, arctiin and arctigenin undergo metabolic transformation mediated by the gut microbiota. In 1992, Nose et al. [10] first confirmed that co-culturing arctiin with mouse fecal microbiota generated arctigenin and 3′-demethylarctigenin (3′-DMAG). In 2003, Xie et al. [11] identified six metabolites, including arctigenin, 3′-DMAG, and enterolactone, in an in vitro co-incubation system of arctiin with gut microbiota. In 2007, Jin et al. [12] isolated the first pure culture strain capable of transforming arctiin from human feces, *Eubacterium* sp. ARC-2, which metabolically converted arctiin into seven derivatives, including 3′-DMAG . In 2013, our research group isolated a strictly anaerobic bacterium, *Blautia* sp. AUH-JLD56, from human feces. Under anaerobic conditions, this strain independently catalyzes the directed conversion of arctiin or arctigenin into 3′-DMAG. When arctigenin was used as the substrate, the maximum conversion concentration reached 3.6 mM, with an average conversion rate as high as 90.5% [13].

The human gut microbiota plays a key role in maintaining host health, and related research has received widespread attention. Developing next-generation probiotics has become a promising technological strategy for regulating the gut microbiota and improving host health. Strictly anaerobic bacteria account for 90%~99% of the gut microbiota and represent the core group of the gut microbial community. However, due to limitations in anaerobic culture conditions and the fact that most strictly anaerobic bacteria rely on synergistic interactions between strains for optimal growth, the availability of gut-specific strains that can be successfully isolated, identified, and functionally characterized remains very scarce. At the same time, the natural source of 3′-DMAG is rare and difficult to obtain, leading to a lack of systematic studies on its related biological activities. Previous research by our group confirmed that, at the same concentration, 3′-DMAG exhibits significantly better DPPH radical scavenging activity than its precursor compound, arctigenin [13]. *In vivo* animal experiments further demonstrated that 3′-DMAG significantly inhibits tumor growth without obvious toxic side effects [6]. Given that the microbial bioconversion products of arctiin and arctigenin possess superior biological activities, this research direction has become a hot topic in the field. However, strictly anaerobic bacteria are highly sensitive to oxygen and cannot grow or proliferate normally under conventional aerobic conditions. Their culture and bioconversion processes both rely on a strictly anaerobic environment, and the anaerobic systems have drawbacks such as high maintenance costs and large equipment investments, which severely limit the functional mechanistic analysis and industrial transformation application of these strains. Therefore, establishing effective strategies to enhance the oxygen tolerance of strictly anaerobic bacteria and achieve their stable growth under aerobic conditions is a key direction for breaking through current research bottlenecks.

Currently, research on improving oxygen tolerance in strictly anaerobic bacteria remains relatively limited. Clostridia, as typical obligate anaerobes, are highly sensitive to oxygen, and existing studies have begun to explore their regulatory mechanisms of oxygen tolerance. In 2007, Riebe et al. [14] demonstrated that the desulfoferrodoxin produced by the obligate anaerobe *Clostridium acetobutylicum* exhibits superoxide reductase activity and is a core functional component for intracellular reactive oxygen species scavenging. In 2008, Hillmann et al. [15] knocked out the

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homologue of the peroxide repressor protein in *C. acetobutylicum*, and the resulting mutant showed a significantly prolonged oxygen tolerance time and could achieve limited growth under aerobic conditions. In 2014, Zhang et al. [16] performed a comparative genomic reconstruction analysis of the Rex regulator in 11 *Clostridium* species, revealing that Rex responds to changes in the intracellular NADH/NAD^+ ratio and regulates downstream gene expression, thereby mediating fermentation product synthesis and oxidative stress tolerance in *C. acetobutylicum*. The strictly anaerobic bacterium *Faecalibacterium prausnitzii* is a core commensal species with high abundance and high detection rate in the human gut and is closely associated with host health. In 2023, the Bäckhed team, while retaining its core probiotic functions, adaptively modified *F. prausnitzii* and successfully endowed the strain with oxygen tolerance; the modified strain could grow syntrophically with the sulfate-reducing bacterium *Desulfovibrio piger* and produce butyrate, providing an important reference for the development of next-generation strictly anaerobic probiotics [17]. Furthermore, our research group previously in 2015, by gradually reducing the concentration of reducing agents in the culture medium and decreasing the depth of liquid medium, domesticated the strictly anaerobic bacterium *Clostridium* sp. AUH-JLC108 isolated from chicken feces into an oxygen-tolerant mutant strain *Clostridium* sp. Aeroto-AUH-JLC108, thereby achieving the efficient synthesis of *O*-desmethylangolensin from daidzein under aerobic conditions [18].

In this study, the strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 [13], previously isolated by our research group, was used as the starting strain. Through continuous oxygen tolerance domestication, a stable oxygen-tolerant mutant strain was obtained. This mutant strain can efficiently catalyze the conversion of arctigenin into the highly active product 3′-DMAG in the presence of atmospheric oxygen, achieving a maximum conversion concentration of 8.2 mM. This study accomplished for the first time the efficient aerobic biosynthesis of 3′-DMAG, overcoming the dependence of the transformation process of this type of strain on a strictly anaerobic environment, and also provides new strategies and insights for the oxygen tolerance domestication and industrial application of gut strictly anaerobic bacteria.

2. Materials and Methods

2.1. Culture Media and Chemicals

Brain Heart Infusion medium (BHI, Becton, Dickinson and Company, USA) was used to cultivate strictly anaerobic intestinal bacteria.

Activation medium: based on BHI medium, supplemented with 0.08% agar, 0.15% L-cysteine, and 0.15% vitamin C. Acclimatization medium: based on the activation medium, the amounts of agar and reducing agents were gradually reduced until the final concentrations of agar, L-cysteine, and vitamin C all reached zero. Agar, L-cysteine, and vitamin C were all purchased from Beijing Solarbio Science & Technology Co., Ltd., China. Arctigenin standard (purity > 98%) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd., China. 3′-Demethylarctigenin standard was prepared using our previously described microbial biotransformation method [13].

2.2. Test Strain

Blautia sp. AUH-JLD56 is a strictly anaerobic bacterium previously isolated by our research group from human fecal samples [13]. The master stock was preserved in a 10% skim milk solution (Inner Mongolia Yili Industrial Group Co., Ltd., China), overlaid with a 2 mm thick layer of liquid paraffin (Tianjin Damao Chemical Reagent Factory, China), and stored in an ultra-low temperature freezer at−80°C.

2.3. Oxygen-Tolerance Domestication Process

The strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 preserved in an ultra-low temperature freezer was inoculated into 4 mL of freshly sterilized BHI medium at an inoculum size of 10% (v/v).

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The culture was incubated in an anaerobic chamber (BAKER Ruskinn, Ruskinn Ltd., UK) under an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 at 37°C for 24 h under static conditions. The pre-cultured broth of the strictly anaerobic strain AUH-JLD56 was then inoculated into the above-mentioned activation medium and incubated at 37°C in a biochemical incubator (ZXDP-A2160, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd., China) for 24 h. This culture process was repeated five times, and the entire procedure was designated as the "first round of acclimatization". After the first round of acclimatization, the substrate arctigenin was added to the culture broth, and the mixture was co-cultured for 3 days. The culture was then extracted with ethyl acetate, evaporated to dryness, redissolved in 100% methanol, and the bioconversion of the substrate arctigenin was analyzed by high-performance liquid chromatography (HPLC). The specific HPLC method is detailed in the section on HPLC detection, preparation, and identification of arctigenin transformation products.

Subsequently, based on the growth yield and growth rate of the acclimatized strain, the concentrations of ascorbic acid, L-cysteine, and agar in the medium were successively reduced in increments of 0.005% to 0.01%. Once the acclimatized strain exhibited stable growth (after five passages), it was transferred to the next gradient of acclimatization medium for further adaptation. This process was continued until the strain was able to grow and transform normally in 4 mL of freshly sterilized BHI medium, at which point the entire acclimatization process was completed. The resulting oxygen-tolerant mutant was designated as the aerobic strain Aeroto-AUH-df6.

2.4. Cell Morphology, Growth Property, Physiological and Biochemical Indicators

The strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 and the oxygen-tolerant strain Aeroto-AUH-df6 were each cultured to the mid-to-late exponential growth phase. The cell morphology of the strains was observed under a light microscope (CN15-T31, KONKYO, China) at 40× magnification. During the same period, the $OD_{600\ nm}$ and pH of the culture broth were measured at fixed time intervals, and the growth curves as well as the change in pH over time were plotted. Biochemical indicator changes before and after oxygen-tolerant domestication were determined using the API20 kit (bioMérieux, Lyon, France). Biochemical tests not included in the kit were performed according to the procedures described in the Wadsworth-KTL Anaerobic Bacteriology Manual [19].

2.5. Determination of the 16S rRNA Gene Sequence of the Oxygen-Tolerant Strain

Genomic DNA of the oxygen-tolerant strain was extracted using a method based on that described by Konstantinos Minas [20], with minor modifications. The 16S rRNA gene sequence was amplified by PCR using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), followed by DNA sequencing. The obtained sequence was aligned with the 16S rRNA sequence of the non-acclimatized strain, and a phylogenetic tree was constructed

2.6. Determination of Oxygen Tolerance of the Oxygen-Tolerant Strain

To investigate the influence of the medium height on bacterial growth, the oxygen-tolerant strain Aeroto-AUH-df6 was inoculated at a 10% inoculum amount into fresh BHI liquid medium with different liquid depths (i.e., liquid medium heights of 2, 1, and 0.75 cm, respectively). Under the same subculture and culture conditions, each setup was subcultured five times. Similarly, to study the effect of inoculum size on the growth of the oxygen-tolerant strain Aeroto-AUH-df6, the strain was inoculated at different inoculum amount (specifically 10%, 5%, 2.5%, and 1.25%) into 4 mL of freshly sterilized BHI medium. Under the same subculture and culture conditions, each setup was subcultured five times. The OD value of the oxygen-tolerant strain Aeroto-AUH-df6 was measured at a UV absorption wavelength of 600 nm. The experiment was repeated three times.

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2.7. HPLC Detection and Structural Identification of Arctigenin Bioconversion Products

After co-culturing the oxygen-tolerant strain Aeroto-AUH-df6 with the substrate arctigenin for 3 days, 1 mL of the culture broth was taken and extracted with an equal volume of ethyl acetate. The extract was evaporated to dryness, reconstituted in 100% methanol, filtered through an organic membrane, and then analyzed by HPLC (Waters 2695 system equipped with a photodiode array detector). The chromatographic column was a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm). Mobile phase A was 10% acetonitrile in water containing 0.1% acetic acid, and mobile phase B was 90% acetonitrile in water containing 0.1% acetic acid. The elution method was A:B = 60:40 (v/v) at a flow rate of 1 mL/min, injection volume 20 μL, and detection wavelength 280 nm. The semi-preparative chromatographic conditions for the product of arctigenin converted by the oxygen-tolerant strain Aeroto-AUH-df6 were as follows: a Kromasil C_{18} preparative column (250 mm × 10 mm, 5 μm), flow rate 2.0 mL/min, and detection wavelength 280 nm. The bioconversion products were analyzed by mass spectrometry (ESI-MS) on a Bruker Daltonics Apex-Ultra mass spectrometer (Massachusetts, USA) and by ^{1}H and ^{13}C NMR spectroscopy in $CDCl_3$ on a Bruker AVANCE NMR spectrometer (400 MHz).

2.8. Determination of the Bioconversion Dynamics of Arctigenin by the Strain Before and After Domestication

10 mL of the strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 culture and the oxygen-tolerant strain Aeroto-AUH-df6 culture were inoculated into 100 mL of fresh BHI liquid medium in an anaerobic chamber and a clean bench, respectively. The substrate arctigenin was added to a final concentration of 0.4 mM. Samples of the anaerobic strain were taken every 4 hours within 24 hours, while samples of the oxygen-tolerant strain were taken every 2 hours within 24 hours. Each sample was tested in triplicate. The reduction of the substrate and the production of the metabolite were detected by HPLC. Curves showing changes in substrate and product concentrations over culture time were plotted for both the anaerobic and the oxygen-tolerant strain cultures.

2.9. Determination of the Transformation Capacity of Arctigenin by the Strain Before and After Acclimatization

The culture broths of the strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 and the oxygen-tolerant strain Aeroto-AUH-df6 were inoculated at a 10% inoculum amount into 4 mL of fresh BHI liquid medium in an anaerobic chamber and a clean bench, respectively, and different concentrations of the substrate arctigenin were added. The final concentrations of arctigenin added to the co-culture with the strictly anaerobic strain AUH-JLD56 in the anaerobic chamber were 0.6 mM, 1.2 mM, 2.4 mM, 3.6 mM, and 4.0 mM. The final concentrations of arctigenin added to the co-culture with the oxygen-tolerant strain Aeroto-AUH-df6 in a conventional biochemical incubator were 4.6 mM, 5.8 mM, 7.0 mM, 8.2 mM, and 9.4 mM. When the final concentration of arctigenin added exceeded 2.5 mM, the substrate was added in 2-4 portions to avoid inhibition of bacterial growth caused by an excessively high single addition. The time interval between two consecutive substrate additions was 4 hours. After 3 days of co-culture, the transformation of the substrate arctigenin was analyzed by HPLC. The experiment was repeated three times.

The calculation formulas for the substrate conversion rate and the average product formation rate

are as follows:

$$R_1 = \frac{C_1}{C_1 + C_2} * 100\% \quad (1)$$

$$R_2 = \frac{C_1 + C_2}{C} * 100\% \quad (2)$$

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R_1: conversion rate; R_2: the average product formation rate; C_1: product concentration (mM); C_2: concentration of the unconverted residual substrate (mM); C: concentration of the substrate added to the medium (mM)

2.10. Statistical Analysis

All data were statistically analyzed using IBM SPSS Statistics 26.

3. Results

3.1. Cell Morphology and Growth Properties of the Oxygen-Tolerant Domesticated Strain

Through oxygen tolerance domestication, we successfully obtained an oxygen-tolerant mutant strain, designated as the oxygen-tolerant strain Aeroto-AUH-df6, from the strictly anaerobic bacterium *Blautia* sp. AUH-JLD56. This oxygen-tolerant strain exhibits significant differences in multiple biological characteristics compared with the wild-type strain *Blautia* sp. AUH-JLD56. Observations under an optical microscope revealed that the wild-type strictly anaerobic strain *Blautia* sp. AUH-JLD56, cultured in an anaerobic chamber, displays oval-shaped cells that mostly exist as single cells, with a few appearing as short rods formed by two connected cells (Figure 1A). During the oxygen-tolerance domestication process, cell length first increased (Figure 1B), then significantly decreased, eventually approaching that of the undomesticated original strain. Ultimately, the obtained oxygen-tolerant strain Aeroto-AUH-df6, when cultured in a conventional biochemical incubator, appears as short rods forming chains of two connected cells (Figure 1C).

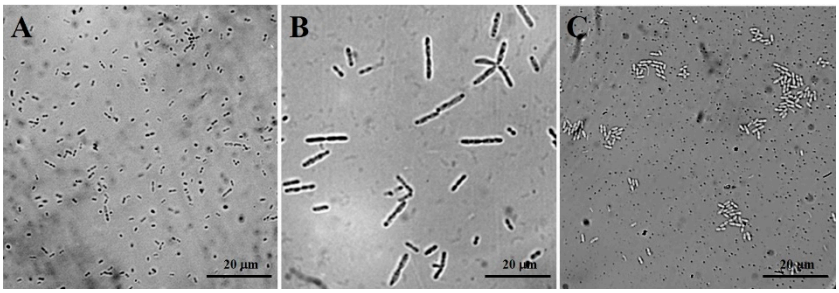

Figure 1. Light micrographs showing (**A**) elliptical cells of the original strictly anaerobic bacterial strain AUH-JLD56 grown anaerobically; (**B**) elongated cells during the oxygen tolerance domestication process; and (**C**) cells of the finally obtained oxygen-tolerant mutant strain Aeroto-AUH-df6 grown aerobically. The bacterial cells were sampled in the late exponential phase.

Compared with the wild-type strain AUH-JLD56 cultured in the anaerobic chamber (Figure 2A, solid line), the oxygen-tolerant strain Aeroto-AUH-df6 cultured in the conventional biochemical incubator showed a significantly higher growth rate. However, the maximum $OD_{600\ nm}$ of this strain averaged 2.37, which was lower than the average maximum $OD_{600\ nm}$ of 2.82 observed for the wild-type strain AUH-JLD56 under anaerobic conditions. In addition, the dynamic characteristics of the growth processes differed markedly: the $OD_{600\ nm}$ of the wild-type strain AUH-JLD56 exhibited a continuous decline after 10 h of inoculation. In contrast, the oxygen-tolerant strain Aeroto-AUH-df6 showed a brief decrease in biomass between 8 and 10 h post-inoculation, followed by a rapid entry into the stationary phase, during which the $OD_{600\ nm}$ did not show any obviously decline (Figure 2B, solid line). Regarding dynamic changes in the pH of the cultural broth, the pH of the wild-type strain AUH-JLD56 continuously decreased from 7.0 to approximately 6.5 within 0~10 h post-inoculation and then remained stable (Figure 2A, dashed line). The pH changes of the oxygen-tolerant strain Aeroto-AUH-df6 displayed multi-stage characteristics: a rapid decrease within 0~2 h post-inoculation, followed by a slight increase over the next 2 h, then a second rapid decrease, ultimately stabilizing at around 5.1 (Figure 2B, dashed line).

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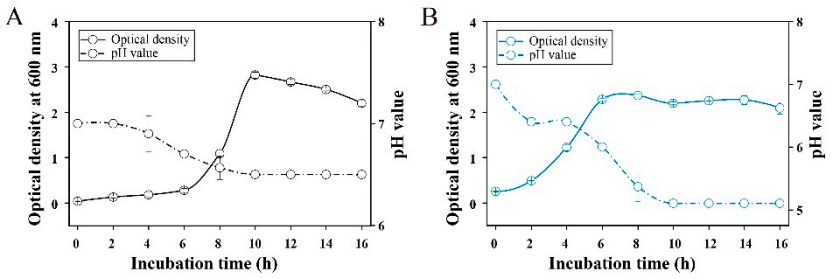

Figure 2. Time course of bacterial cell growth (solid line) and pH change (dashed line) for the original strictly anaerobic strain AUH-JLD56 grown anaerobically (**A**) and the oxygen-tolerant mutant strain Aeroto-AUH-df6 grown aerobically (**B**). The inoculum concentration was 10% (v/v). The culture broth of each strain was sampled every 2 h to measure both pH and OD600. Error bars represent the standard deviation of biological replicates.

3.2. Variation of Physiological and Biochemical Indicators Before and After Domestication

Before domestication, the strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 exhibited negative urease activity. After oxygen tolerant domestication, the resulting mutant strain Aeroto-AUH-df6 showed positive urease activity. Compared with the original strain *Blautia* sp. AUH-JLD56, the oxygen-tolerant strain Aeroto-AUH-df6 demonstrated significantly enhanced carbon source metabolic utilization capability. The original strain AUH-JLD56 was unable to utilize sucrose, xylose, raffinose, rhamnose, arabinose, mannose, trehalose, melezitose, cellobiose, glycerol, salicin, or sorbitol, whereas the oxygen-tolerant strain Aeroto-AUH-df6 acquired the ability to metabolize and utilize all of the above carbon sources (Table 1).

Table 1. Physiological and biochemical indicators of the original strictly anaerobic wild-type strain *Blautia* sp. AUH-JLD56 and the oxygen-tolerant mutant strain Aeroto-AUH-df6

Identification	Strain AUH-JLD56	Strain Aeroto-AUH-df6	Identification	Strain AUH-JLD56	Strain Aeroto-AUH-df6
Urease	–	+	Arabinose	–	+
Amylohydrolysis	–	–	Mannose	–	+
Indole production	–	–	Trehalose	–	+
H2S production	–	–	Melezitose	–	+
Glucose	–	–	Cellobiose	–	+
Maltose	–	–	Gelatin	–	–
Sucrose	–	+	Esculin	–	–
Xylose	–	+	Glycerol	–	+
Raffinose	–	+	Salicine	–	+
Rhamnose	–	+	Sorbitol	–	+

3.3. 16S rRNA Gene Sequence Analysis of the Oxygen-Tolerant Domesticated Strain

The 16S rRNA gene of the oxygen-tolerant strain Aeroto-AUH-df6 was sequenced, and a phylogenetic tree was constructed together with the original non-domesticated strain AUH-JLD56 (Figure 3). BLAST sequence alignment showed that the oxygen-tolerant strain Aeroto-AUH-df6 shared 99.43% sequence similarity with the original strain *Blautia* sp. AUH-JLD56, and 99.01% similarity with *Blautia* wexlerae strain WAL 14507. Phylogenetic analysis indicated that this oxygen-tolerant strain clustered within the same evolutionary branch as *Blautia* genus strains. In conclusion, the mutant strain obtained through oxygen-tolerant domestication was identified as belonging to the genus *Blautia* and named *Blautia* sp. Aeroto-AUH-df6.

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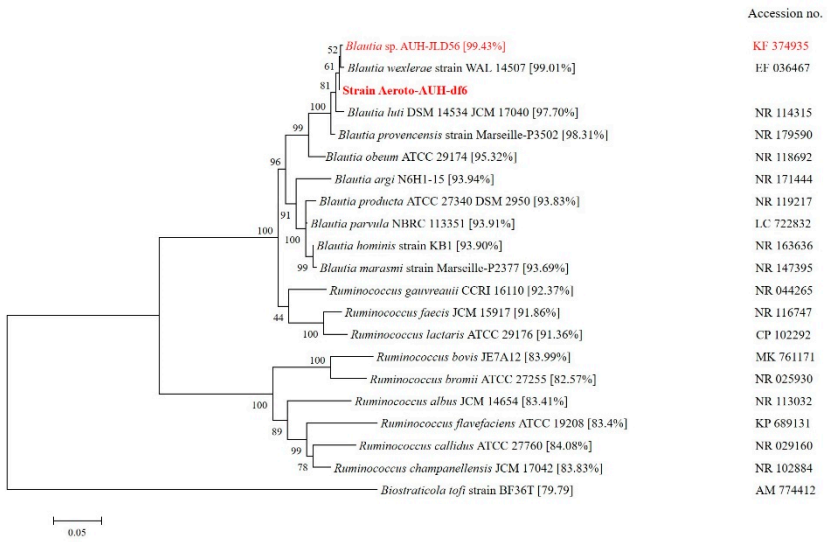

Figure 3. Phylogenetic tree showing the relationship of the oxygen-tolerant strain Aeroto-AUH-df6 to related taxa based on 16S rRNA gene sequences.

3.4. Analysis of Oxygen Tolerance of the Oxygen-Tolerant Domesticated Strain

Equal amounts of the oxygen-tolerant strain Aeroto-AUH-df6 were inoculated into liquid media with different liquid layer heights (2 cm, 1 cm, 0.75 cm). The results showed that the liquid layer height had no significant effect on the mean maximum $OD_{600\,nm}$ of this oxygen-tolerant mutant strain (Figure 4A). Further investigation into the effect of different inoculum sizes on strain growth revealed that even when the inoculum amount was reduced to 1.25%, the oxygen-tolerant strain Aeroto-AUH-df6 could still proliferate stably after five consecutive passages (Figure 4B), indicating that reducing the inoculum size did not significantly inhibit the growth performance of this strain.

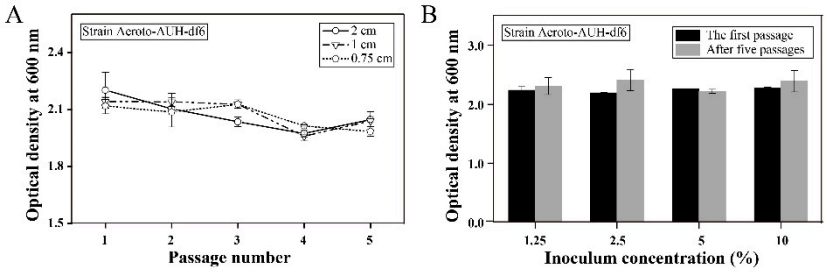

Figure 4. Influence of culture depth (**A**) and inoculum concentration (**B**) on cell growth of the oxygen-tolerant strain Aeroto-AUH-df6 in the presence of atmospheric oxygen. Cultures with a final OD600 of 2.0 were used as inoculum. Growth yield was determined every 8 hours, followed by continuous passaging for five generations. Error bars represent the standard deviation of biological replicates.

3.5. Identification of the Conversion Product of Arctigenin by the Oxygen-Tolerant Domesticated Strain

After co-culturing the oxygen-tolerant strain Aeroto-AUH-df6 with the substrate arctigenin, the extract of the culture broth was analyzed by HPLC. The results showed that, in addition to the residual unconverted substrate arctigenin, a new characteristic chromatographic peak was detected at a retention time of 8.70 min (Figure 5). This chromatographic peak exhibited maximum UV absorption at 227 nm and 280 nm, which was completely consistent with the UV absorption characteristics of the demethylated product of arctigenin, 3′-DMAG (Figure 6A). Further mass spectrometry analysis of this new chromatographic peak was performed in negative ion mode (Figure 6B), yielding an ion peak signal of ESI^-: m/z 357 ($[M-H]^-$), corresponding to a molecular weight of 358. This matches exactly the theoretical molecular weight of the standard compound

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3′-DMAG (molecular formula $C_{20}H_{22}O_6$). In conclusion, based on the HPLC retention time, UV absorption spectrum, and mass spectrometry data, the metabolite with a retention time of 8.70 min was identified as 3′-DMAG.

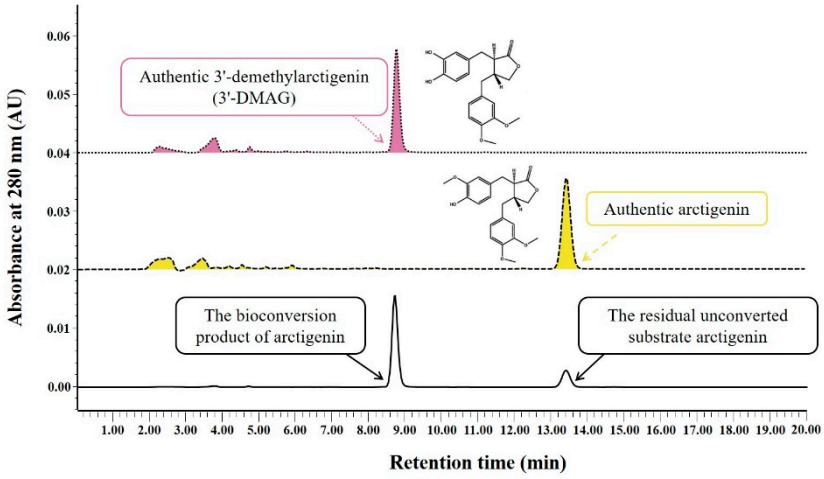

Figure 5. High-performance liquid chromatography (HPLC) elution profiles of arctigenin metabolism by the oxygen-tolerant strain Aeroto-AUH-df6 in the presence of atmospheric oxygen.

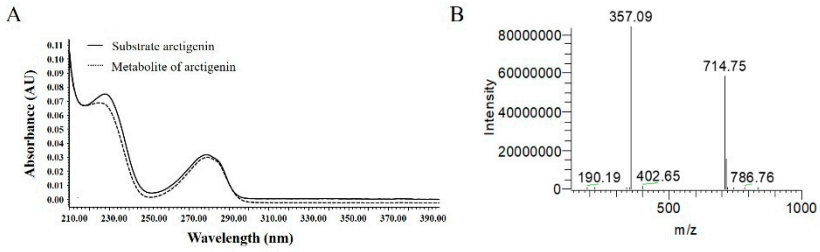

Figure 6. UV spectrum (**A**) and mass spectrum (**B**) of the arctigenin metabolite produced aerobically by the oxygen-tolerant strain Aeroto-AUH-df6.

To further clarify the chemical structure of this new compound peak, this study performed proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) characterization analyses on the purified target component. The detailed results are as follows:

^{1}H-NMR (CDC_{13}, 400 MHz,): δ 2.46-2.64 (4H, m, H-2, 3, 7″), 2.85 (2H, d, J=5.5 Hz, H-7′), 3.81-3.84 (6H, s, -OCH3*2), 3.88 (1H, dd, J = 8.54, 7.6 Hz, H-4), 4.13 (1H, dd, J= 8.54, 7.8 Hz, H-4), 6.48 (1H, d, J=1.94 Hz, H-2″), 6.51 (1H, dd, J=8.19, 1.94 Hz, H-6′), 6.57 (1H, dd, J=8.19, 1.94 Hz, H-6″), 6.66 (1H, d, J=1.94 Hz, H-2′), 6.75 (1H, d, J=8.19 Hz, H-5′), 6.77 (1H, d, J=8.19 Hz, H-5″).

^{13}C-NMR (CDC_{13}, 400 MHz): δ 179.9 (C-1), 149.0 (C-3″), 147.8 (C-4″), 144.0(C-3′), 142.9 (C-4′), 130.0 (C-1′), 129.9 (C-1′), 121.7 (C-6′), 120.7 (C-6″), 116.1 (C-2′), 115.2 (C-5′), 111.8 (C-2″), 111.4 (C-5″), 71.8 (C-4), -Me 55.9, -Me 55.8, 46.6 (C-2), 40.9 (C-3), 38.2 (C-7″), 34.0 (C-7′)。

Through combined interpretation of the ^{1}H-NMR and ^{13}C-NMR data, the target metabolite with a retention time of 8.70 min was precisely identified as 3′-DMAG. This result confirms that the oxygen-tolerant strain Aeroto-AUH-df6, obtained through oxygen-tolerance domestication process, can efficiently catalyze the conversion of the substrate arctigenin to 3′-DMAG under aerobic conditions in a conventional biochemical incubator.

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3.6. Bioconversion Dynamics of the Oxygen-Tolerant Domesticated Strain

The strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 and the oxygen-tolerant strain Aeroto-AUH-df6 were separately co-cultured with the substrate arctigenin in an anaerobic chamber and a conventional biochemical incubator, respectively. Samples were taken at different time points to dynamically monitor the substrate conversion process. The results showed that for the strictly anaerobic strain AUH-JLD56, the concentration of arctigenin decreased rapidly after 4 h of inoculation; during the period of 8~12 h post-inoculation, the concentration of the product 3′-DMAG increased rapidly in a linear manner, after which the rate of increase tended to level off (Figure 7A). In contrast, for the oxygen-tolerant strain Aeroto-AUH-df6, during the first 4~6 h post-inoculation, the substrate arctigenin was consumed rapidly in a linear fashion, while the product 3′-DMAG was simultaneously generated rapidly and linearly; thereafter, both the rate of substrate consumption and product synthesis slowed down significantly (Figure 7B).

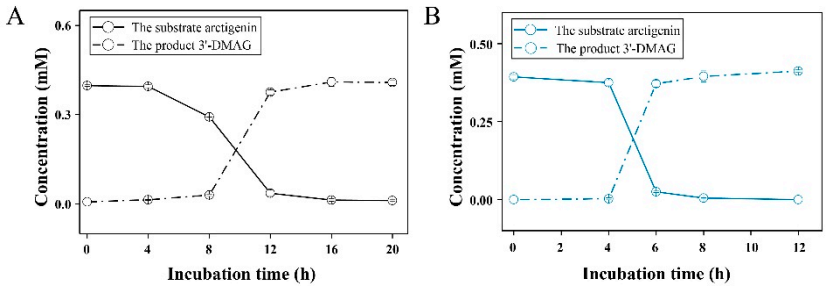

Figure 7. Bioconversion kinetics of the substrate arctigenin by the original strictly anaerobic strain AUH-JLD56 grown anaerobically (**A**) and by the oxygen-tolerant mutant strain Aeroto-AUH-df6 grown aerobically (**B**). The inoculum concentration was 10% (v/v). The initial concentration of arctigenin was 0.4 mM.

3.7. Conversion Capacity of the Oxygen-Tolerant Strain Toward the Substrate Arctigenin

To clarify the difference in conversion efficiency toward arctigenin before and after oxygen-tolerance domestication, the original strictly anaerobic strain *Blautia* sp. AUH-JLD56 and the oxygen-tolerant strain Aeroto-AUH-df6 were separately inoculated into pure BHI medium in an anaerobic chamber and a conventional biochemical incubator, respectively, and co-cultured with different concentrations of arctigenin. After 3 days of culture, the substrate conversion efficiency was determined by HPLC. The results showed that the conversion capacity of the non-domesticated wild-type strain AUH-JLD56 (Figure 8A) toward arctigenin was significantly inferior to that of the oxygen-tolerant strain Aeroto-AUH-df6 (Figure 8B). Under anaerobic conditions, the upper concentration limit for efficient conversion of arctigenin by the wild-type strain was 3.6 mM, at which the average substrate conversion rate was 90.09% and the average product formation rate was 83.31%.

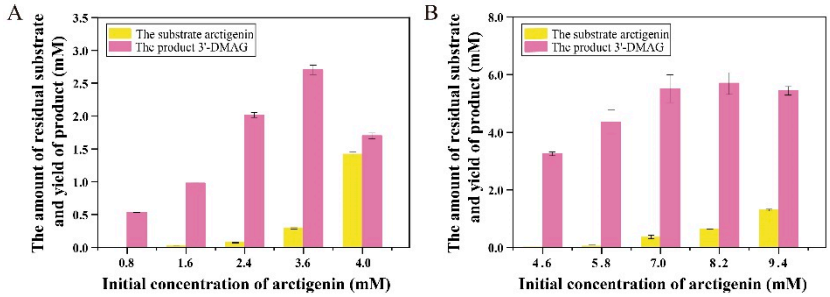

Figure 8. Bioconversion capacity of the original strictly anaerobic strain AUH-JLD56 grown anaerobically (**A**) and of the oxygen-tolerant mutant strain Aeroto-AUH-df6 grown aerobically (**B**). Compound 3′-DMAG is the metabolite of the substrate arctigenin.

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Under aerobic culture conditions, when the final concentration of arctigenin was ≤5.8 mM, the substrate was almost completely converted to 3′-DMAG. When the substrate concentration was increased to 7.0 mM, the average substrate conversion rate and average product formation rate of the oxygen-tolerant strain were 93.75% and 83.99%, respectively. At a concentration of 8.2 mM, the average conversion rate and formation rate were 90.01% and 77.15%, respectively. When the substrate concentration was further increased to 9.4 mM, the conversion capacity of the strain decreased significantly, with the average conversion rate dropping to 80.70% and the average product formation rate to 71.61%. In conclusion, under aerobic conditions, the upper concentration limit for efficient conversion of arctigenin by the oxygen-tolerant strain Aeroto-AUH-df6 was 8.2 mM.

4. Discussion

Arctium lappa L. fruit is a traditional Chinese medicinal herb with complex chemical constituents, among which the lignan compound arctiin is its core characteristic active ingredient. Since traditional Chinese medicines are mostly administered orally, arctiin enters the body and is preferentially degraded by the gut microbiota in the intestine, generating various derivatives. In 2001, Heinonen et al. [21] first demonstrated that human fecal microbiota possess the ability to metabolize arctiin, converting it into enterolactone and other active metabolites. In 2013, our research group isolated a strictly anaerobic bacterium, *Blautia* sp. AUH-JLD56, from a human fecal sample. This strain can sequentially deglycosylate arctiin under anaerobic conditions to produce arctigenin, and further specifically convert arctigenin into 3′-DMAG. Our *in vitro* antioxidant experiments showed that in the concentration range of 0.025~0.100 mM, 3′-DMAG exhibited significantly stronger DPPH radical scavenging activity than its precursor arctigenin ($p < 0.01$) [13]. Subsequent studies also revealed that 3′-DMAG had a significantly stronger inhibitory effect on human hepatocellular carcinoma HepG2 cells *in vitro* than arctigenin. Furthermore, using a mouse xenograft model of liver cancer, we found that 3′-DMAG significantly suppressed tumor growth without obvious toxic side effects [6]. The metabolite 3′-DMAG of arctigenin possesses excellent bioactivity, but its large-scale preparation by chemical synthesis is currently not feasible. The isolation of strain *Blautia* sp. AUH-JLD56 offers a possible approach for the microbial transformation synthesis of 3′-DMAG. However, this wild-type strain is strictly anaerobic and highly sensitive to oxygen; both its growth and substrate conversion require strict anaerobic conditions. This oxygen sensitivity greatly limits its industrial application.

To address the above problem, this study used the strictly anaerobic bacterium *Blautia* sp. AUH-JLD56 as the starting strain, conducted long-term oxygen tolerance acclimation, and successfully obtained an oxygen-tolerant mutant strain, named *Blautia* sp. Aeroto-AUH-df6. Compared with the wild-type strain AUH-JLD56, this oxygen-tolerant mutant exhibited significant changes in cell morphology, physiological and biochemical characteristics, substrate conversion rate, and maximum conversion capacity. Under anaerobic conditions, the wild-type strain AUH-JLD56 efficiently converted arctigenin, with a maximum conversion concentration of 3.6 mM, an average conversion rate of 90.09%, and a product yield of 83.31%. In contrast, the oxygen-tolerant mutant Aeroto-AUH-df6 efficiently converted arctigenin under aerobic conditions, achieving a maximum conversion concentration of 8.2 mM, with an average conversion rate of 90.01% and a product yield of 77.15%. Notably, when the final concentration of arctigenin was ≤ 7.0 mM, the product yield of the oxygen-tolerant mutant was essentially consistent with that of the wild-type strain under anaerobic conditions; when the substrate concentration was increased to 8.2 mM, the average conversion rate did not decrease significantly, but the product yield dropped to 77.15%; when the substrate concentration was further increased to 9.4 mM, the product yield further decreased to 71.61%. Our research group previously obtained an oxygen-tolerant mutant strain *Clostridium* sp. Aeroto-AUH-JLC108, capable of C-ring cleavage of soy isoflavone daidzein under aerobic conditions. Studies have confirmed that under high-concentration daidzein (2.4 mM) stress, this strain forms a protective film structure on the cell surface, mainly composed of polysaccharides [18]. This structure not only acts as a physical barrier against oxidative damage but also slows down substrate permeation into the cells, thereby alleviating the toxic damage caused by high substrate

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concentrations. Based on this, we speculate that under high-concentration (e.g., 8.2 mM) arctigenin stress, the oxygen-tolerant mutant Aeroto-AUH-df6 may similarly form a protective structure on its cell surface.

To verify the above speculation, this study cultured the oxygen-tolerant mutant Aeroto-AUH-df6 in a conventional biochemical incubator, using cells without arctigenin addition as a control, and performed Congo red staining analysis on cells exposed to a high concentration (8.2 mM) of arctigenin. The results showed that, compared with cells without arctigenin (Figure 9A), the outer wall of cells treated with high-concentration arctigenin did not form an obvious protective film structure (Figure 9B). We speculate that this phenomenon may be related to the relatively low toxic damage caused by 8.2 mM arctigenin to the cells. Our previous studies have confirmed that 5 mM arctigenin and its metabolite 3′-DMAG exhibit no antibacterial activity against *Staphylococcus aureus* (ATCC27217), *Salmonella paratyphi* (CMCC50001), or *Escherichia coli* (CICC10372) [13]. In the present study, when the final concentration of arctigenin was 7.0 mM, the strain achieved an average substrate conversion rate of 93.75% and a total average yield of substrate and product of 83.99%, which was essentially consistent with the conversion level of the wild-type strain under anaerobic conditions, further indicating that 7.0 mM arctigenin and its conversion product 3′-DMAG did not cause obvious toxic damage to Aeroto-AUH-df6. Based on the changes in product yield, when the substrate concentration was increased to 8.2 mM and 9.4 mM, the product yield showed a significant decreasing trend, suggesting that the substrate concentration at 8.2 mM already exerted some toxicity on the cells. However, because the toxic stress intensity was limited, the protective film structure formed by the cells was not prominent. From the perspective of compound structure, the product has one more hydroxyl group than the substrate, suggesting that the water solubility of the product may be higher than that of the substrate. If so, the amounts of unconverted substrate and unexported product retained in the protective film may not be equal; instead, the substrate with lower water solubility would account for a higher proportion. To test this hypothesis, after the reaction, we centrifuged the culture medium, collected the cells for homogenization, and compared the substrate and product contents in the culture supernatant and the cell homogenate using HPLC. The HPLC detection results precisely confirmed the above hypothesis: the substrate peak was very large and the product peak was very small in the cell samples, which was completely opposite to the results in the supernatant (Figure 10). Finally, we measured the maximum water solubility of the substrate and product at 37 °C, and the results were as expected: the solubility of the substrate (arctigenin) was 0.153 g/L, and the maximum solubility of the product was 0.870 g/L (not yet reported). In short, the higher water solubility of the product allows it to more easily “escape” from the protective film, while the substrate is more likely to be “trapped” inside the protective film.

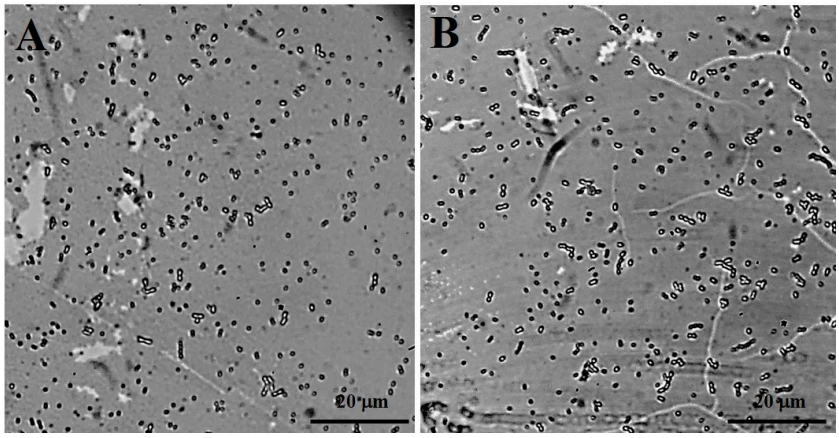

Figure 9. Light micrographs of the derived oxygen-tolerant strain Aeroto-AUH-df6 stained with Congo Red. Bacterial cells were sampled after 3 days of incubation in a conventional biochemical incubator under conditions without (**A**) or with (**B**) 8.2 mM arctigenin.

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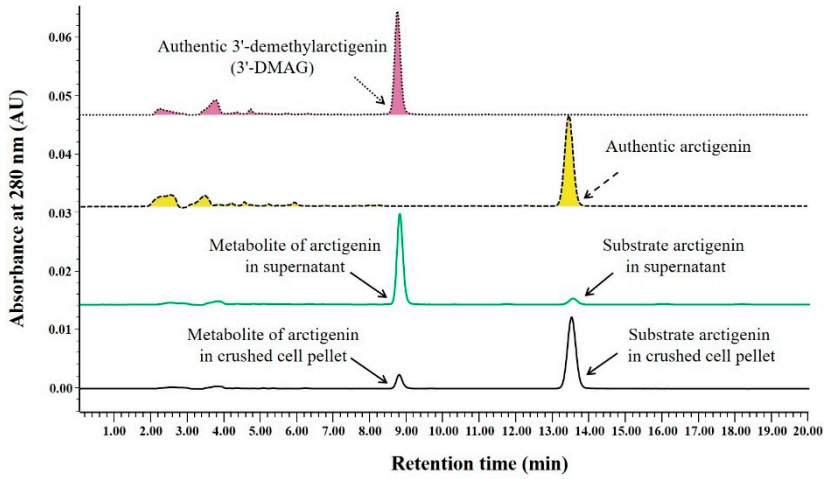

Figure 10. HPLC elution profiles of the substrate arctigenin and its metabolite 3′-DMAG extracted from the culture supernatant (**A**) and from the crushed cell pellet (**B**).

In addition, compared with the original strain AUH-JLD56, the oxygen-tolerant mutant Aeroto-AUH-df6 acquired the ability to utilize sucrose, xylose, raffinose, rhamnose, arabinose, mannose, trehalose, melezitose, cellobiose, glycerol, salicin, and sorbitol. Notably, despite this significant broadening of the carbon source utilization spectrum, the biomass of the oxygen-tolerant mutant under aerobic conditions ($OD_{600\ nm}$ = 2.37) did not increase; rather, it was lower than that of the wild-type strain under anaerobic conditions ($OD_{600\ nm}$ = 2.82). We also observed that, compared with the original wild-type strain, the oxygen-tolerant mutant caused a continuous decrease in the pH of the culture medium during aerobic cultivation, eventually stabilizing at around 5.1 (Figure 2). To analyze the organic acid metabolic characteristics of this strain under aerobic conditions, the culture broth was analyzed by HPLC, and the results showed that the main metabolite was acetic acid (Figure 11). These results suggest that the oxygen-tolerant mutant may redirect its carbon metabolic flux toward an acidogenic pathway to cope with aerobic stress, rather than using it for cell proliferation.

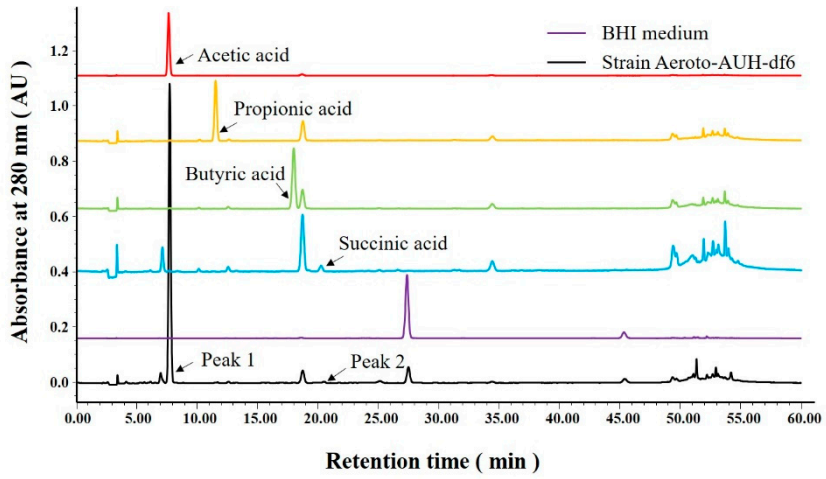

Figure 11. HPLC elution profile of short-chain fatty acids produced in the culture medium of the oxygen-tolerant strain Aeroto-AUH-df6.

Although acetic acid itself has no direct antioxidant activity, it can indirectly regulate intracellular redox homeostasis. As early as 1971, O'Brien and Morris [22] first demonstrated that the key strategy of *Clostridium acetobutylicum* to achieve short-term oxygen tolerance is the continuous

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synthesis of acetic acid: acetate helps maintain intracellular reducing power balance and eliminates oxygen toxicity, thereby ensuring short-term survival of the bacterium under aerobic stress. In 2002, Karnholz et al. [23] further found that some strictly anaerobic acetogenic bacteria can synthesize acetate via the acetyl-CoA pathway to adapt to low-oxygen environments. In 2007, Briukhanov and Netrusov [24] systematically reviewed the stress effects of aerobic environments on strictly anaerobic bacteria and clearly identified acetogenesis as the main metabolic strategy for short-term oxygen tolerance in obligate anaerobes, a process that achieves adaptive regulation to aerobic stress by maintaining redox balance and coupling with reactive oxygen species scavenging mechanisms. In the present study, the oxygen-tolerant mutant Aeroto-AUH-df6 actively redirected its carbon metabolic flux, prioritizing the use of carbon sources for acetate synthesis to alleviate intracellular oxidative stress. This "metabolic trade-off" mechanism well explains why the mutant exhibited a significantly broadened carbon source utilization spectrum while its biomass did not increase but rather decreased: carbon sources and energy were largely used for acetate production to combat oxygen toxicity, rather than for growth and reproduction. How this mutant regulates acetate synthesis under aerobic conditions remains to be further investigated.

5. Conclusions

Through long-term oxygen tolerance domestication of the strictly anaerobic wild-type *Blautia* sp. AUH-JLD56, an oxygen-tolerant mutant strain was successfully obtained. Phenotypic analysis showed that the growth of the mutant under aerobic conditions was slightly lower than that of the wild-type under strict anaerobic conditions. Compared with the wild-type, the mutant exhibited an accelerated aerobic growth rate and enabled stable conversion of arctigenin. Moreover, the maximum concentration of arctigenin converted by the mutant under aerobic conditions (8.2 mM) was significantly higher than that achieved by the wild-type (3.6 mM). The oxygen tolerance domestication method developed in this study achieves aerobic adaptive modification while preserving the demethylation conversion function of the strain, which is of great value for promoting the aerobic development and industrial application of gut strict anaerobes with specific bioconversion functions.

Author Contributions: Conceptualization, X.W., W.Y. and M.L.; methodology, X.W., W.Y. and M.L.; validation and formal analysis, W.Y., M.L., H.J. and Z.Z.; data curation, W.Y.; writing—original draft preparation, W.Y.; writing—review and editing, X.W. and H.L.; visualization, W.Y., H.J. and Z.Z.; supervision, X.W. and H.L.; project administration, X.W.; funding acquisition, X.W. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

3′-DMAG	3′-demethylarctigenin
HPLC	high-performance liquid chromatography

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