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Evaluating the impact of redox potential on the growth capacity of anaerobic gut fungi

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Abstract

Anaerobic gut fungi (AGF, *Neocallimastigomycota*) inhabit the alimentary tract of herbivores. Although strict anaerobes, studies have suggested their capacity to retain viability after various durations of air exposure. It is currently unclear whether AGF can actively grow, and not merely survive, in redox potentials (E_h) higher than those encountered in the herbivorous gut. We evaluated the growth of two AGF strains (*Orpinomyces joyonii* and *Testudinimyces gracilis*) at various E_h levels, achieved by manipulating the concentrations of reductant (cysteine hydrochloride) in culture media. Both strains exhibited robust and sustainable growth at negative E_h (−50 mV or below). However, growth in the absence of cysteine hydrochloride (E_h value around +50 mV) was possible only for *O. joyonii* and only for one subcultivation. The capacity to grow at +50 mV was further confirmed in four additional taxa (*Pecoramyces ruminatium*, *Anaeromyces mucronatus*, *Aklioshbmyces papillarum*, and *Piromyces communis*), while two (*Aestipascuomyces dupliciliberans* and *Capellomyces foraminis*) failed to grow under these conditions. Our results establish the ability of AGF to grow at redox potential values higher than those encountered in their natural habitats. Such capability could contribute to efficient AGF dispersal and horizontal transmission between hosts, and could have important implications for industrial applications of AGF.

Keywords: anaerobic fungi; redox potential; anaerobiosis

Introduction

Herbivores depend on microorganisms residing in their alimentary tracts to break down plant biomass. A wide range of microorganisms (bacteria, archaea, protozoa, and fungi) inhabit the alimentary tract of herbivores (Gruninger et al. 2014). Fungi in the herbivorous gut belong to a distinct phylum (*Neocallimastigomycota*) and have been shown to play a crucial role in the depolymerization of the plant polysaccharides cellulose and hemicellulose (Hess et al. 2020). Anaerobic gut fungi (AGF) are ubiquitous in wild and domesticated ruminant, pseudo-ruminant, and hindgut mammalian herbivores (Meili et al. 2023). Recently, their detection and isolation from various tortoises (family *Testudinidae*) was also reported (Pratt et al. 2023, 2024).

All AGF strains identified so far are strict anaerobes (Hanafy et al. 2022) that lack respiratory capacities and rely on a mixed acid fermentation scheme for energy generation (Borneman et al. 1989, Akhmanova et al. 1999, Huang et al. 2018). This characteristic, unique compared to all members of the kingdom Fungi, is a reflection of their oxygen-free natural habitat (the rumen and hindgut of herbivores), where a highly negative redox potential (E_h −250 to −350 mV) prevails (Huang et al. 2018). However, an extreme sensitivity to air exposure and a restrictive obligate dependency on highly reduced conditions would impair AGF survival outside of their host, and hence negatively impact animal-to-animal transmission through the oral–fecal route. Further, air is transiently introduced to the alimentary tract of herbivores through the feeding cycle (Huang et al. 2018). As such, capacity for survival at higher redox potential could improve AGF transmission and survivability.

A limited number of studies exist on the response of AGF to air exposure. Viability of the AGF strains *Pecoramyces ruminantium* strain C1A (Struchtemeyer et al. 2014), *Neocallimastix* sp. (Milne et al. 1989), or *Neocallimastix* and *Caecomyces* spp. (Leis et al. 2014) to air by sparging in a sealed culture (Milne et al. 1989), or aliquoting a small volume into a sterile Petri dish (Leis et al. 2014) was assessed. In all studies, cultures remained viable after various levels of air exposure. This sustained viability could partly be attributed to oxygen scavenging by reductants in the media in both cases. Further, the aerotolerance capabilities of AGF have further been corroborated in studies where AGF were recovered from fecal samples dried and exposed to air under different field and laboratory conditions (Orpin 1981, Milne et al. 1989, Brookman et al. 2000, Hanafy et al. 2020). Such abilities, in addition to microscopic observation of specific structures that do not correspond to various life cycle stages of AGF (Fig. 1) have led to speculations that these are aerotolerant structures produced in response to oxygen exposure (Hanafy et al. 2022, Orpin 1981, Milne et al. 1989, Brookman et al. 2000, Hanafy et al. 2020).

However, while these studies indicate a potential capacity of AGF to survive air exposure, it is currently unknown whether AGF can actively grow, and not merely survive, in conditions where redox potentials are higher (more positive) than those encountered in the herbivorous gut (E_h of −250 to −350 mV) (Huang et al. 2018), or in highly reduced anaerobic media where reducing agents such as cysteine hydrochloride (henceforth CystHCl) retains E_h around −200 to −250 mV. Quantifying the growth capacity of AGF representatives under various redox potential regimes would aid in

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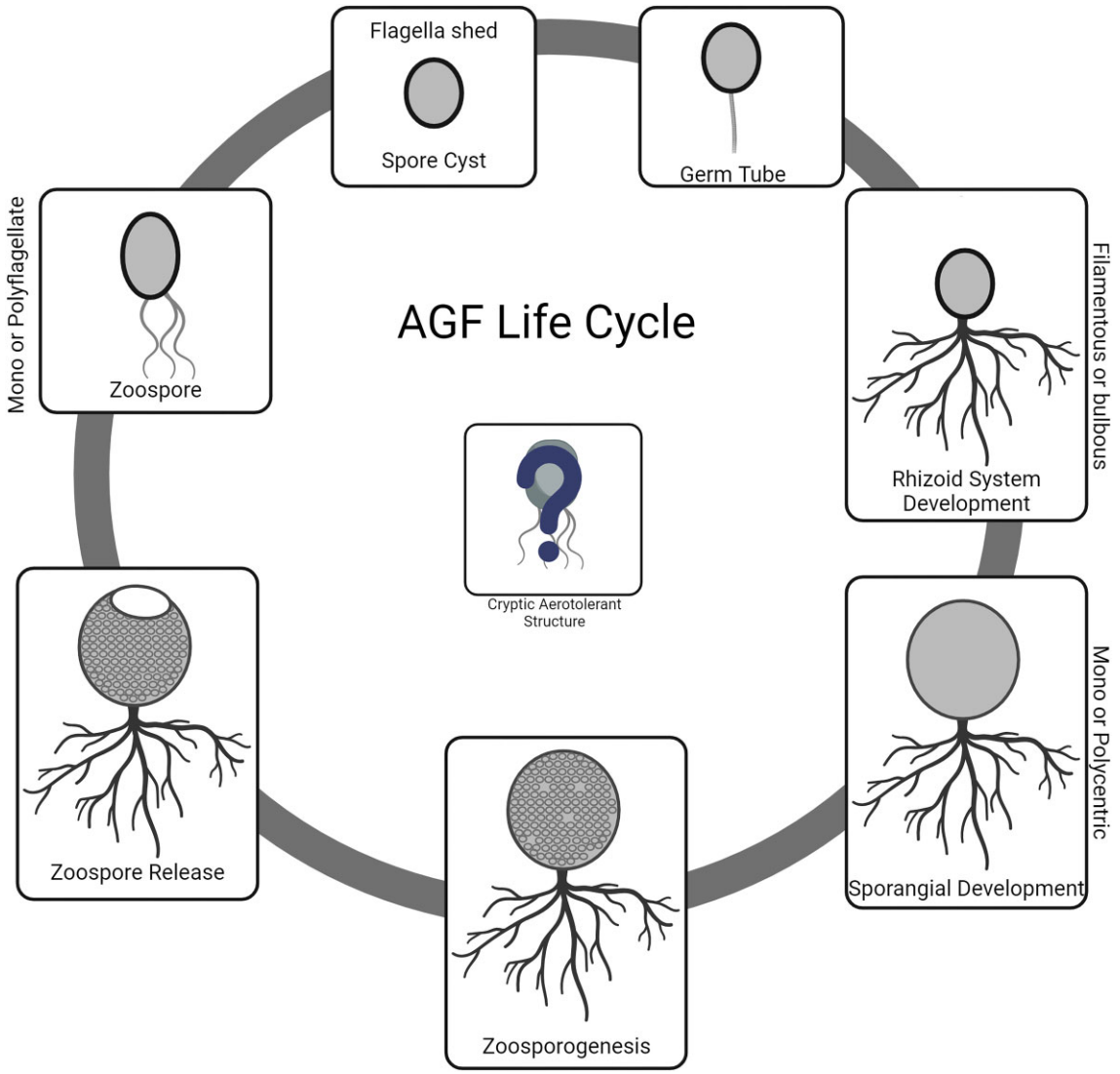

Figure 1. Growth cycle of AGF. The possible existence of yet-unidentified resting stage/aerotolerant structures (depicted by a "?") has been proposed but not conclusively demonstrated yet.

Table 1. AGF isolates evaluated in this study.

Isolate	Genus	Origin	Rhizoidal growth pattern	Hyphal growth	Reference
AB.3	*Orpinomyces joyonii*	American bison	Polycentric	Filamentous	(Hanafy et al. 2022)
T130A.3	*Testudinimyces gracilis*	Ploughshare tortoise	Polycentric	Filamentous	(Pratt et al. 2023)
orc.30	*Pecoramyces ruminatium*	Oryx	Monocentric	Filamentous	This study
can.4	*Anaeromyces mucronatus*	Cow	Polycentric	Filamentous	This study
AP1.1	*Piromyces communis*	Alpaca	Monocentric	Filamentous	This study
Cap.2A	*Capellomyces foraminis*	Barbary sheep	Monocentric	Filamentous	This study
SR1.3	*Aestipascuomyces dupliciliberans*	Barbary sheep	Monocentric	Filamentous	This study

gauging scalability and formulating operational parameters in industrial settings. Here, we sought to quantify the ability of AGF to grow at different redox potentials. We show that AGF strains can grow and retain viability at E_h of −50 mV. Further, we demonstrate that many AGF strains can grow at a positive redox potential (+50 mV) in initial subcultures, but that such capacity could not be sustained upon subsequent subculturing.

Materials and methods

Cultures

AGF strains examined in this study are listed in Table 1. All strains were maintained in rumen fluid-cellobiose (RFC) media amended with antibiotics (50 μg/ml penicillin, 20 μg/ml streptomycin, and 50 μg/ml chloramphenicol) to inhibit the growth of bacteria as previously described (Calkins et al. 2016). Ten milliliter was

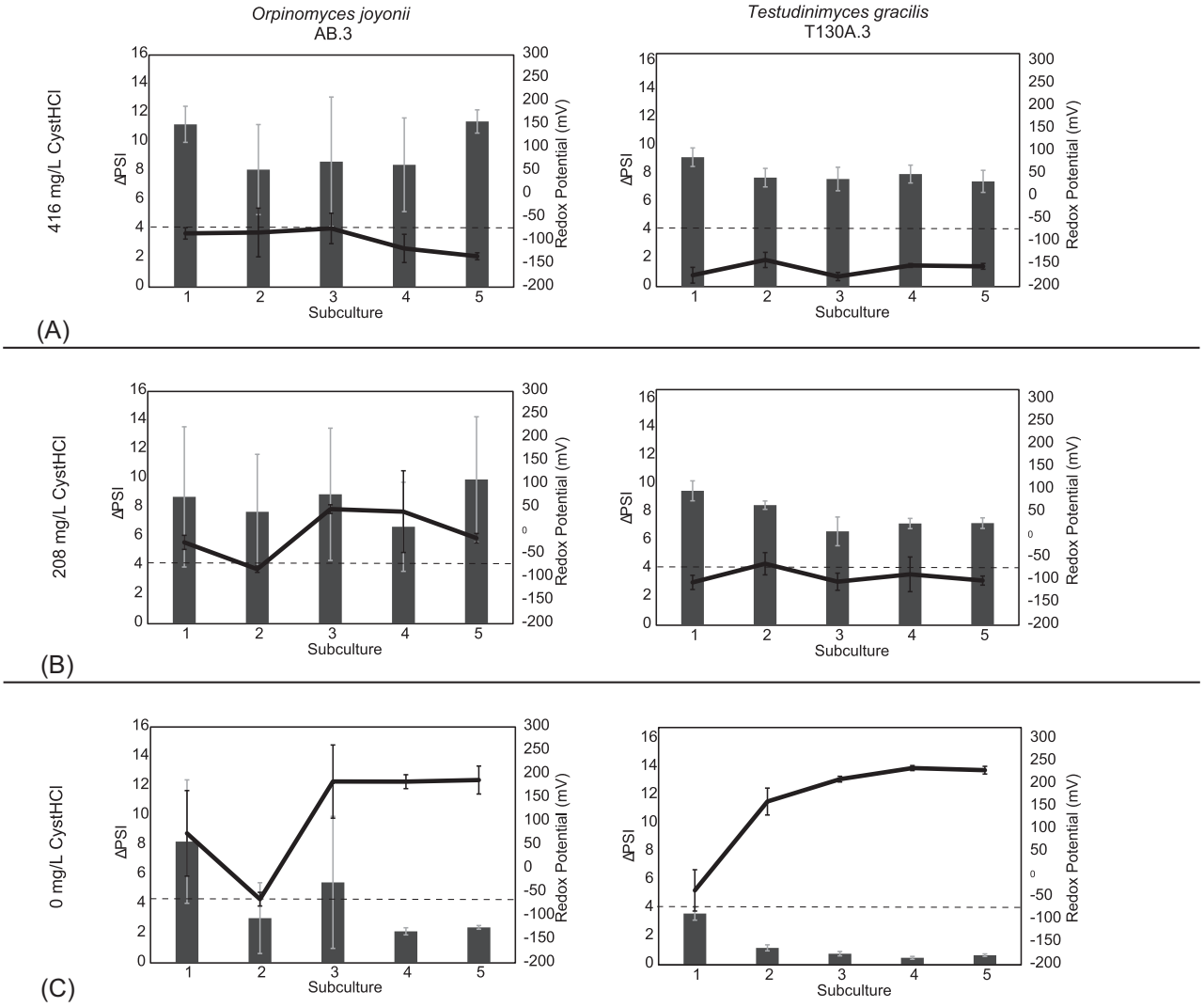

Figure 2. Growth of AGF strains *Orpinomyces joyonii* strain AB.3 (left column) and *Testudinimyces gracilis* strain T130A.3 (right column) in media with 416 (A), 208 (B), or 0 mg/l (C) CystHCl. The bars in each graph correspond to the average (from four replicates) headspace gas pressure (ΔPSI) (primary Y-axis) used here as a proxy for growth (gray error bars showing the standard deviation), and the thick line corresponds to the average (from four replicates) redox potential E_h (secondary Y-axis) measured at the conclusion of each subculture (black error bars showing the standard deviation). The dashed line shows the cutoff headspace gas pressure value (ΔPSI = 4) we used to consider the growth positive. The number of subcultures are shown on the *X*-axis. The interval between subcultures was 4 days for *Orpinomyces joyonii* strain AB.3 and 7 days for *Testudinimyces gracilis* strain T130A.3.

dispensed into anaerobic tubes (Balch tubes) under a stream of CO_2, and tubes were sealed using butyl rubber stoppers and aluminum crimp seals. Mammalian-sourced strains were maintained at 39°C and sub-cultured twice weekly, while tortoise-sourced strains were maintained at 30°C and sub-cultured once weekly, as previously described (Brookman et al. 2000).

Experimental set up

To assess the growth of AGF strains at different redox potentials, RFC media was prepared in 10 ml aliquots in Balch tubes without the addition of CystHCl, the reductant used for lowering redox potential in RFC media used for regular subculturing and maintenance of AGF. CystHCl was then added from a sterile anoxic 1.25% stock solution at various volumes (0.4 ml, 0.1 ml, or none per 10 ml) to achieve a final concentrations of approximately 416, 208, or 0 mg/l. Redox potential was measured in media using an Orion benchtop meter, equipped with a Thermo Scientific Orion 9179BNMD Redox/ORP Electrode.

Growth of AGF at all three CystHCl concentrations was initially assessed in one mammalian-sourced and one tortoise-sourced strain in quadruplicates. *Orpinomyces joyonii* strain AB.3 was isolated from an American bison (Hanafy et al. 2022). The genus *Orpinomyces* produces polycentric thalli and filamentous hyphae, belongs to the family *Neocallimastigaceae*, and represents one of the most ubiquitous and abundant genera encountered in fecal and rumen samples of mammalian herbivores (Meili et al. 2023). *Testudinimyces gracilis* strain T130A.3 was isolated from a ploughshare tortoise, and represents the most common genus encountered in tortoise fecal samples examined so far (Pratt et al. 2023). In addition, we tested the growh of five additional strains (Table 1) under one condition (0 mg/l CystHCl) to determine the prevalence of the unexpected observed capacity to grow under this condition in the AGF.

Cultures used for initial inoculation into the three CystHCl concentrations were grown in media with 0.4 ml CystHCl per tube (416 mg/l). A 3 ml inoculum of fully grown culture was used for different subcultures. Prior to being used as inoculum, all regular

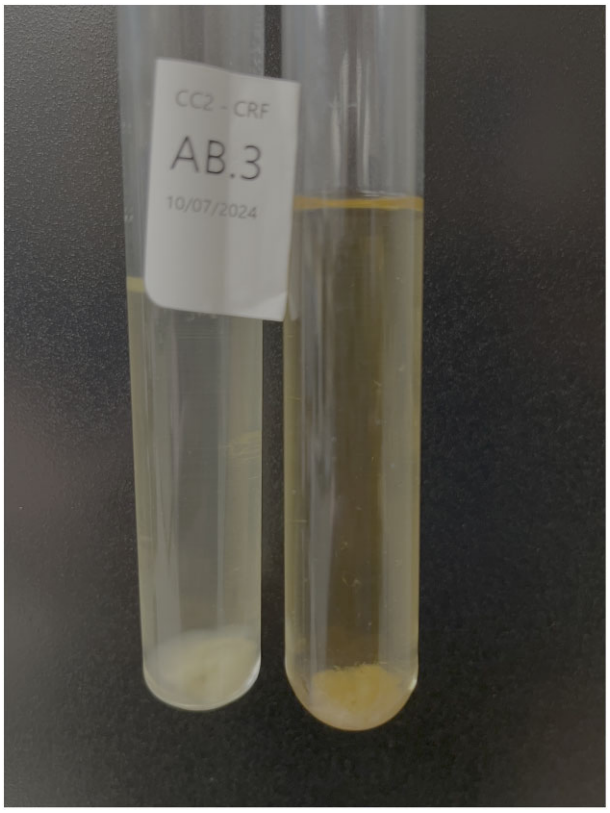

Figure 3. Photograph documenting the ability of *Orpinomyces joyonii* strain AB.3 to grow in both fully reduced media with 416 mg/l HCl (left), as well as non-reduced media without CystHCl. The orange color is formed due to the blending of yellow color of rumen fluid, and red/oxidized color of as evident by the oxidized (orange, a mix of yellow from rumen fluid and red, the color of the oxidized from of resazurin).

media was removed, and media at the designated CystHCl concentration was added to minimize carry-over of regular CystHCl-containing media. Redox potential of uninoculated media, as well as the culture media at the end of each subculture was measured. Cultures were grown to reach the stationary phase (4 days for mammalian-sourced strains and 7 days for tortoise-sourced strains) before subculturing into media with the same CystHCl concentration. Growth of AGF strains was assessed visually, as well as by measuring headspace pressure, generated from the production of CO_2 and H_2 gases as part of AGF mixed acid fermentation metabolism (Borneman et al. 1989, Akhmanova et al. 1999, Boxma et al. 2004). A gas pressure of >4 PSI was deemed positive. Assessment of gas pressure has previously been used as a proxy for fungal growth quantification (Theodorou et al. 1994, Wilken et al. 2020). Gas pressure was measured using a pressure transducer (SSI technologies Inc., Janesville, WI, USA).

Results

AGF growth capacity at different level of redox potentials

Uninoculated media prepared with CystHCl concentration of 416 mg/l had a redox potential of −171 ±13 mV ($n = 3$) prior to inoculation. Redox potential at the conclusion of the first subculture in both strains examined ranged between −180 to −350 mV (Fig. 2A); and remained steady (mostly within −80 to −300 mV range) throughout subsequent subcultures (Fig. 2A). Both strains exhibited strong growth (gas pressure of 8–10 for *Orpinomyces joyonii* strain AB.3, and 7- 9 PSI for *Testudinimyces gracilis* strain T130A.3) throughout the first and all subsequent subcultures (Fig. 2A). Such a pattern of robust growth is expected, given that these conditions are used for routine maintenance of these strains in our laboratory.

Uninoculated media prepared with CystHCl concentration of 208 mg/l had a redox potential of −48 ±3 mV ($n = 3$) prior to inoculation. Redox potential at the conclusion of the first subculture ranged from −120 mV to +0 mV and, broadly speaking, remained steady, mostly within the −100 to 0 mV range throughout subsequent subcultures (Fig. 2B). Interestingly, both strains tested also exhibited strong growth at this slightly elevated (more positive) level of redox potential, as evident by gas production (gas pressure up to 10 PSI for *Orpinomyces joyonii* strain AB.3, and 10 PSI for *Testudinimyces gracilis* strain T130A.3) and visual inspection. Gas pressure readings for *Orpinomyces joyonii* strain AB.3 or for *Testudinimyces gracilis* strain T130A.3 did not differ significantly in the presence of 208 mg/l versus 416 mg/l CystHCl (Student's *T*-test *P*-values > 0.5).

Media prepared with no CystHCl (0 g/l) had a redox potential of 152 ± 19 mV ($n = 3$). Such value is more positive than the redox potential of the resazurin (resorufin/dihydroresorufin indicator pair E_h of −51 mV) used in the media, as evident by the oxidized color in the Balch tubes. Redox potential at the first subculture ranged from −20 mV to +60 mV and slowly increased to the 100–200 mV range through subsequent subcultures. Interestingly, *Orpinomyces joyonii* strain AB.3 grew in the first subculture, as evident by gas production (PSI values of 8.23). Visual inspected also confirmed the growth of strain AB.3 in media without CystHCl (Fig. 3). On the other hand, the tortoise-sourced *Testudinimyces gracilis* strain T130A.3 did not grow in the absence of CystHCl (Fig. 2C) as evident by lack of gas production and visual inspection.

Finally, we examined six additional AGF strains, to assess the prevalence of the observed ability of AGF strains to grow at a positive E_h. Out of the six strains examined, four (*Pecoramyces ruminatium* strain orc.30, *Piromyces communis* strain AP1.1, *Anaeromyces mucronatus* strain can.4, and *Aklioshbmyces papillarum* strain wts.1) demonstrated such capacity (Fig. 4), with *Aklioshbmyces papillarum* showing highest level of growth and gas production. On the other hand, two (*Aestipascuomyces dupliciliberans* strain SR1.3, and *Capellomyces foraminis* strain Cap.2A) failed to grow under such conditions (Fig. 4).

Discussion

Here, we tested the growth of AGF strains at three different redox potential regimes. Our results revealed that AGF strains can grow at redox potential levels (−50 mV, 208 mg/l CystHCl) that are higher (more positive) than typically encountered in their natural habitat [−250 to −350 mV (Huang et al. 2018)], as well as routinely used in media for their maintenance (Fig. 2B). More interestingly, we demonstrate that many, but not all, AGF can grow at an even higher redox potential (+50 mV) (Figs 2 and 4), but could not be sustained in subsequent subculturing efforts. AGF genomes encode genes putatively involved in protection against oxidative stress, e.g. superoxide dismutase and glutathione peroxidase. These genes were shown to be differentially expressed in *Pecoramyces ruminantium* upon air exposure (Struchtemeyer et al. 2014). However, it is currently unknown whether the same genes would also be expressed during growth at a higher redox potential, and a more thorough investigation is needed, for example follow-

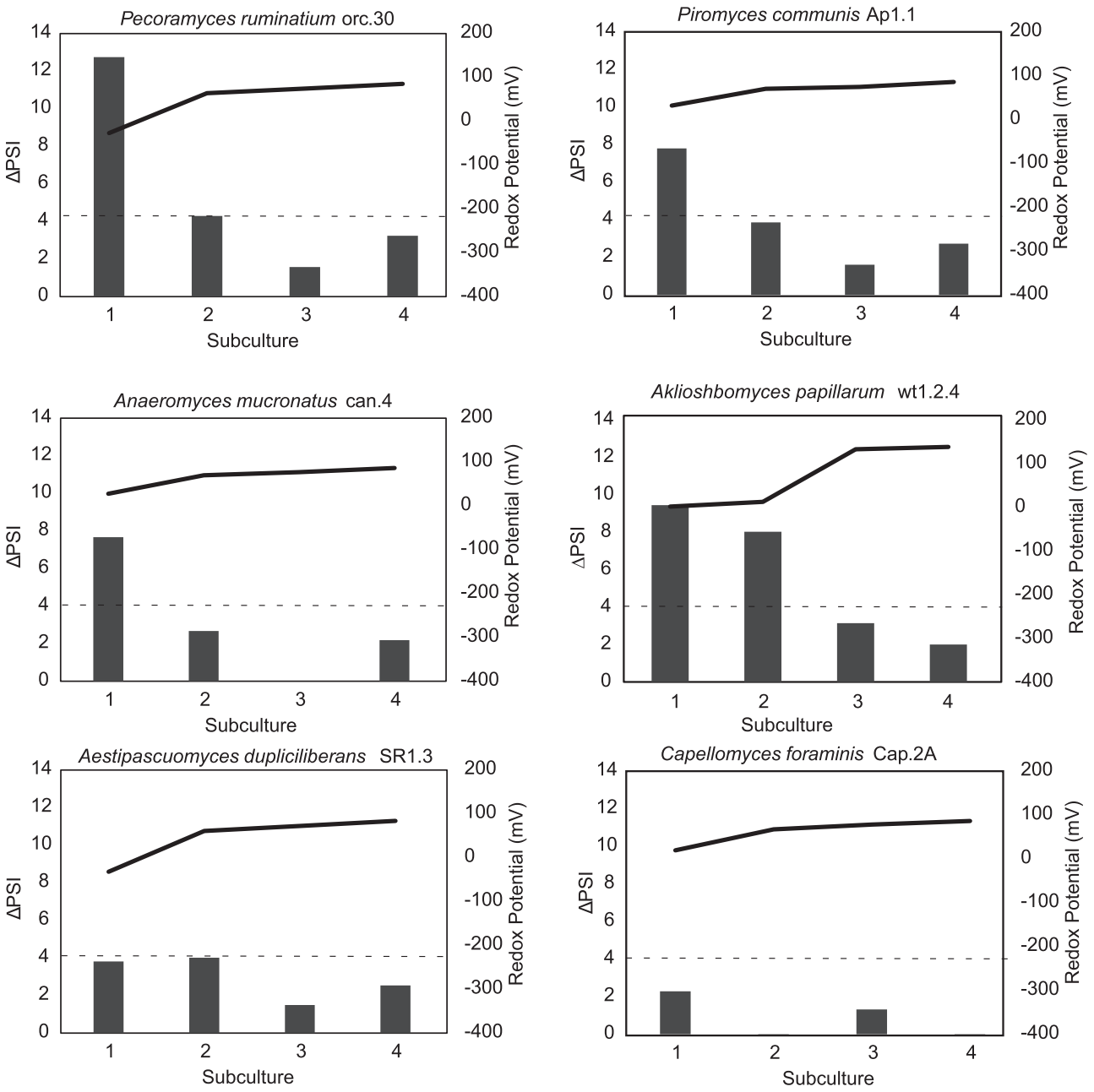

Figure 4. Growth of AGF strains *Pecoramyces ruminatium* strain orc.30, *Piromyces communis* strain AP1.1, *Anaeromyces mucronatus* strain can.4, *Aklioshbmyces papillarum* strain wts.1, *Aestipascuomyces dupliciliberans* strain SR1.3, and *Capellomyces foraminis* strain Cap.2A in media with 0 mg/l CystHCl. The bars in each graph correspond to the pressure (ΔPSI) (primary *Y* axis) used here as a proxy for growth, and the thick line corresponds to the redox potential E_h (secondary *Y* axis) measured at the conclusion of each subculture. The dashed line shows the cutoff gas pressure value (ΔPSI = 4) we used to consider the growth positive. The number of subcultures are shown on the *X*-axis. The interval between subcultures was 4 days.

ing the expression of these and other genes using RT-qPCR similar to (Struchtemeyer et al. 2014). Also, characterization of the capability of more AGF strains to grow under similar conditions is needed to map growth at higher redox potential on phylogeny, and to provide a better scheme for finding robust strains combining superior growth and product formation with superior oxygen tolerance.

As described above, while prior studies have focused on assessing aerotolerance of pure cultures of AGF (Milne et al. 1989, Leis et al. 2014, Struchtemeyer et al. 2014), knowledge about the ability of various AGF to grow under different E_h regimes has been lacking. In addition to expanding our basic understanding of physiological preferences, these results could provide interesting clues regarding the global ubiquity and ecological success of AGF in colonizing the alimentary tracts of their herbivorous hosts. The observed capability for sustained growth at −50 mV (208 mg/l CystHCl), as well as the observed ability for growth in positive E_h (Figs 2C, 4) could aid in enhancing their transmissibility between animal species through the oral-fecal route, especially in non-domesticated hosts, where animal-to-animal contact is less common than in domesticated settings. This success is manifested in the fact that AGF are ubiquitous in all herbiv-

orous species that possess a rumen, pseudorumen, or enlarged hindgut. As well, examining a large number of samples from an AGF-harboring species, e.g. cattles, and horses, invariably detects the presence and activity of AGF within each sample. Further, the observed capacity to grow under a relatively wider range of E_h could be a reflection of the capacity of AGF to inhabit various parts of the amimals GIT, where differences in redox potential exists, e.g. between the upper layers of rumen which may periodically be exposed to high redox conditions due to ingestion of feed particles, compared to the center of rumen where low redox conditions permanently exist due to the high levels of microbial activities.

In addition, such observed capacities can be important for AGF scaling efforts in industrial settings. From an applied perspective, AGF possess powerful carbohydrate active enzyme machineries which mobilize and depolymerize cellulose and hemicellulose in plant biomass, rendering AGF extremely promising agents for the production of biofuels, bioproducts, and biogas from plant biomass (Saye et al. 2021). Extreme oxygen sensitivity and obligate requirement for a highly reduced environment has been regarded as a hinderance for scaling AGF cultures in industrial settings. Our results suggest that the AGF redox requirement is not significantly different from that observed with strict anaerobes commonly used for production of value-added chemicals and biofuels [e.g. *Clostridium carboxidivorans* and *Clostridium acetobutylicum* (Zhang et al. 2014, Han et al. 2020, Grimalt-Alemany et al. 2021)], and might open new doors for similar applications for AGF at a larger scale. However, it must be noted that this is only one of multiple hurdles faced while attempting to grow AGF. Lack of reliable storage procedures, propensity to senesce, the requirement for complex growth media for some strains, the relatively slower growth rate, their complex life cycle, and their out-competition in mixed digestor cultures are additional issues that need to be addressed.

Author contributions

Emma E. England (Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Carrie J. Pratt (Data curation, Formal analysis, Investigation, Methodology), Mostafa S. Elshahed (Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing), and Noha H. Youssef (Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing)

Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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