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The administration of L-cysteine and L-arginine inhibits biofilm formation in wild-type biofilm-forming yeast by modulating *FLO11* gene expression

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ABSTRACT

Microbial biofilms are undesired in food manufacturing, drinking water distribution systems, and clinical realms. Yeast biofilms are particularly problematic because of the strong capacity of yeast cells to adhere to abiotic surfaces, cells and tissues. Novel approaches have been developed over recent years to prevent the establishment of microbial biofilms, such as through the use of small molecules with inhibiting and dispersing properties. Here, we studied the inhibitory activity of 11 different amino acids on the biofilm formation ability of three wild-type *S. cerevisiae* strains and the reference strain ∑1278b. Subsequent evaluation of different concentrations of the two most effective amino acids, namely arginine and cysteine, revealed that they acted in different ways. Arginine prevented biofilm formation by reducing *FLO11* gene expression; its addition did not affect cell viability and was even found to enhance cell metabolism (vitality marker) as determined by Phenotype Microarray (PM) analysis. On the contrary, the addition of cysteine reduced both cell viability and vitality as well as *FLO11* expression. Thus, the use of cysteine and arginine as agents against biofilm formation can be diversified depending on the most desired action towards yeast growth.

Keywords: flor yeast; adhesion to plastic; phenotype microarray; RealTime PCR; *FLO11*

INTRODUCTION

Biofilms are generally undesired, especially within the clinical realm, where microorganisms can colonize devices and implants, such as dental prostheses, heart valves, orthopedic implants, etc. (Tan et al. 2014), as well as various host tissues (Kumamoto and Vinces, 2005, Gow et al. 2012). The growth of adverse biofilms has also been reported in food processing and drinking water distribution systems (Abdallah et al. 2014, Gomes et al. 2014). In contrast, microbial biofilms can be beneficial in a plethora of biotechnological processes. For instance, huge potentials lie in the clean-up of hazardous waste sites, the filtering of biofuels and wastewaters, and the formation of bio-barriers to protect soil and groundwater from contamination (Ashraf et al. 2014). They are also crucial in many food processes, for example, in the maturation of cheeses and sausages (Licitra *et* al. 2007, Feofilova et al. 2013, Giaouris et al. 2014), and in the biological aging of wine (Budroni et al. 2005, Legras et al. 2016).

The formation of a biofilm is a cellular response to nutritional signals that allows yeast cells to modify their morphology and metabolism and adapt to environmental changes. In particular, the activation of *FLO11* transcription, through a network that comprises the cAMP/PKA, MAPK and TOR pathways, is essential for biofilm formation as well as pseudohyphal and invasive growth in yeast (Vinod et al., 2008; Zara et al., 2012). The availability of ammonium, or specific amino acids when used as sole nitrogen sources, modulates *FLO11*-dependent phenotypes (Lorenz and Heitman, 1998; Braus et al., 2003; Zara et al., 2011). For example, Gimeno et al. (1992) showed that pseudohyphal differentiation and invasive growth are responses to the availability of proline. Lorenz and Heitman (1998) observed pseudohyphal growth on proline and glutamine-based medium. Bou Zeidan et al. (2014) reported the reduction of biofilm formation by *S. cerevisiae* when L-histidine was added as the sole nitrogen source. Similarly, Szafranski-Schneider et al. (2012) found that L-histidine modulates biofilm formation in *Candida albicans*. Moreover, methionine, valine, and phenylalanine have all been found to induce pseudohyphal morphology in *Pichia fermentans* (Sanna et al. 2012).

In view of these observations, amino acids are therefore promising molecules for the modulation of biofilm formation in yeasts. In the present study, we tested the activity of 11 different amino acids on three wild-type biofilm-forming *S. cerevisiae* strains and the reference strain ∑1278b by evaluating the dose-response relationship, biofilm formation, and cell growth. The activities of cysteine and arginine, the two most promising amino acids against biofilm formation, were further investigated through analysis of *FLO11* expression and the phenotype microarray (PM) technique.

66 **MATERIALS AND METHODS**

67 *Yeast strains, media and culture conditions*

68 The *S. cerevisiae* yeast strains used in this study are reported in **Table 1**. A9, M23 and V80 flor
69 strains were isolated from "Arvisionadu", "Malvasia di Bosa", and "Vernaccia di Oristano" wines,
70 respectively, in Sardinia, Italy, and differ in their *FLO11* gene lengths and expression levels (Zara et
71 al. 2009). These strains are deposited in the culture collection of the University of Sassari (Italy),
72 associate member of the Microbial Resource Research Infrastructure – Italian Joint Research Unit
73 (www.mirri-it.it). *Σ*1278b strain is a standard background for studying filamentous growth and
74 biofilm formation (Granek and Magwene 2010) and its inclusion facilitates comparisons of the
75 results obtained in this and previous studies. Yeast strains were cultured in YPD medium (1% Yeast
76 extract, 2% Peptone, 2% Glucose), 20% YPD medium (1% Yeast extract, 2% Peptone, 20%
77 Glucose), SC medium (0.17% Yeast Nitrogen Base without ammonium sulfate and amino acids,
78 0.5% ammonium sulfate, 2% glucose), flor medium (0.17% of Yeast Nitrogen Base without
79 ammonium sulfate and amino acids, 0.5% ammonium sulfate, 4% ethanol) and in Biolog specific
80 IFY-0 medium (1x IFY-0 culture medium, 20 mM D-glucose, 5 mM KH_2PO_4, 2 mM $NaSO_4$, and
81 1x DyeD Biolog). Cells were prepared as follows: after an overnight incubation in 5 mL YPD at
82 25°C and with 200 rpm agitation, aliquots of the cultures were inoculated into fresh YPD and
83 incubated for 4 h under the same culture conditions to reach the exponential phase (0.4 to 0.5 values
84 of optical density at 600 nm, OD600), washed, suspended in sterile water to the desired
85 concentration, and inoculated into the different media under investigation.

86 *Dose-response analysis*

87 Dose-response assays were carried out according to Bou Zeidan et al. (2014) in 96-well microtiter
88 plates. Briefly, $5x10^4$ cells/mL were inoculated into microtiter wells containing SC medium and
89 varying concentrations (0 to 40 mM) of a single amino acid as the sole nitrogen source. The amino
90 acids investigated were: L-arginine, L-lysine, L-histidine, L-phenylalanine, L-tryptophan, L-
91 threonine, L-proline, L-serine, L-leucine, and L-valine. The microtiter plates were incubated under
92 agitation at 30°C for 48 h in SC medium and growth was measured automatically every 30 min at
93 OD_{600} using a *SPECTROsta*r nano-microplate spectrophotometer (BMG Labtech, Ortenberg,
94 Germany). The DMFit software (Institute of Food Research, Norwich, UK), based on the Baranyi
95 model (Baranyi and Roberts, 1994), was used to fit growth curves and obtain growth parameters.
96

97 *Biofilm formation and cell viability.*

98 Biofilm formation on plastic was evaluated as previously described (Reynolds & Fink, 2001), but

with some modifications as described by Bou Zeidan et al. (2014). Aliquots of 100 µL of cell suspensions containing $5x10^6$ cells/mL in flor medium supplemented with 10 mM L-arginine, L-lysine, L-histidine, L-phenylalanine, L-tryptophan, L-threonine, L-proline, L-serine, L-leucine, L-cysteine, or L-valine were dispensed into 96-well polystyrene microtiter plates (Costar 3595, Corning, NY). Cell suspensions were incubated statically at 30°C for 48 h. Then, an equal volume of 1% (w/v) crystal violet was added to each well. After 30 min, the wells were washed with sterile water and cell adherence to the well walls was quantified by solubilizing the retained crystal violet in 100 µL 10% (w/v) SDS and an equal volume of sterile water. After 30 min, 50 µL of these solutions were transferred into fresh 96-well polystyrene microtiter plates and the A_{570} then measured spectrophotometrically. Cell viability was determined following serial dilution by spot tests and plate counts on YPD agar plates. For the spot test and plate count assays, a duplicate of the biofilm formation assay was used and serial dilutions (1/10) were set by spotting 10 µL of cell suspension after solubilizing the biofilm. The effect of 2 mM, 5 mM, 10 mM, and 20 mM L-arginine and L-cysteine concentrations, each added separately, on yeast adherence was evaluated as described above.

Quantitative Real time PCR.

Yeast cells were inoculated ($5x10^6$ cells/mL) into SC medium supplemented with 5 mM L-arginine or L-cysteine and incubated for 48 h at 25°C (no agitation). Three independent biological replicates were analyzed for each sample. Cells were collected by centrifugation and kept at -80°C until processed for RNA isolation. Total RNA was extracted using the Aurum^{TM} Total RNA Mini Kit (Bio-Rad, Milan, Italy). Two micrograms of total RNA were retrotranscribed using the iScript^{TM} cDNA synthesis kit (Invitrogen Life Technologies, Milan, Italy). Quantitative real time PCR (qPCR) was performed using a PikoReal Real-Time PCR System (Thermo Scientific, Milan, Italy) using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific Milan, Italy) with the primer pairs reported in **Table 2**. The thermal profile was as follows: UDG pre-treatment (50°C for 2 min); activation step (95°C for 10 min); amplification step (40 cycles of: 95°C for 15 s; 58°C for 10 s; and 72°C for 30 s); melting curve program (95°C for 10 s, 60°C for 15 s, 95°C with a heating rate of 0.1°C/s); and cooling step (40°C for 30 s). Differences in *FLO11* transcription levels among different samples were assessed by evaluating sample-specific efficiencies, as proposed by Rao et al. (2013). As suggested by Derveaux et al. (2010), three housekeeping genes were evaluated. The housekeeping genes *ACT1*, *ARF1*, and *SUM1* were selected as those with the greatest transcription stability in flor strains (Zara et al. 2008), as determined by three different validation programs (Jacob et al. 2013).

Phenotype microarray

The phenotype microarray (PM) analysis was carried out using PM3B microtiter plates purchased from Biolog, Omnilog (Hayward, CA, USA), which allowed the screening of 95 different nitrogen sources, including single amino acids, di/tripeptides, purines, etc. (Bochner 2001). Yeast strains were grown on YPD agar plates overnight at 25°C and resuspended in 15 mL nutrient supplement solution using a sterile cotton swab. Cell density was adjusted to 62% transmittance on a Biolog turbidimeter, as equivalent to an OD_{600} of 0.22 (2-3 x10^6 cells/mL). The final inoculating fluids were prepared by diluting the cell suspension 48-fold (62% transmittance in nutrient supplement solution) in IFY-0 apposite culture medium supplemented with 5mM L-Arginine or 5mM L-cysteine. Then, 100 µL of the final inoculating fluids were seeded onto the Biolog PM3B plate and incubated statically at 30°C in an Omnilog Reader for 96 h. Each experiment was performed in duplicate. The quantitative color changes were recorded automatically for each well every 15 min using a CCD camera.

Statistical analyses

If not otherwise stated, statistical analyses were performed in triplicate (at least) using independent cultures. A linear mixed effect analysis of the relationship between growth and amino acid concentration was carried out using R (R Core Team, 2016) and the "lme4" package (Bates et al., 2015). In the model, the fixed effect was the amino acid concentration, while intercepts for strains and by-strain random slopes for the effect of amino acid concentration were the random effects. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. P-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without considering the effect in question. The kinetic responses of the strains obtained from the Omnilog Reader were analyzed using Omnilog-PM software (Biolog, Inc., Hayward, CA, USA) as well as R (R Core Team, 2016) and the "opm" package (Lea et al., 2013). The function do_aggr in the opm package was used to calculate curve parameters from the kinetic raw data via spline-fitting; the function heat_map form the same package allowed the clusterization of observations and variables using the complete linkage method and the Euclidean distance measure.

RESULTS

Dose-response analysis in the presence of amino acids

Dose-response analysis showed that the L-amino acids histidine, arginine, lysine, and cysteine exerted a growth inhibition effect on all the strains with an MIC of 40 mM for histidine and 10 mM

for arginine, lysine, and cysteine. No growth inhibition was observed for any of the tested concentrations of serine, leucine, phenylalanine, proline, threonine, valine, or tryptophan (**Figure 1**).

Biofilm formation in the presence of amino acids

In all the *S. cerevisiae* strains tested, biofilm formation was significantly reduced by histidine, arginine, lysine, and cysteine when added at concentrations ≤ MIC (**Figure 2**). Tryptophan, phenylalanine, and threonine showed limited growth inhibition activity. On the contrary, biofilm formation was enhanced by serine, valine, and proline (always used singularly) in a strain-dependent manner. Overall, the inhibitory effects of arginine and cysteine on biofilm formation were the most potent across all the tested strains; these two amino acids were therefore selected for future analysis.

Biofilm formation at different arginine and cysteine concentrations.

To evaluate better the effect of different concentrations of arginine and cysteine on biofilm formation, 0, 2, 5, 10, and 20 mM cysteine or arginine were added to the biofilm-inducing medium. Both amino acids reduced biofilm formation in a similar manner (**Figure 3**). Arginine affected adherence to plastic ($\chi2(1)=8.4713$, $p=0.0036$), lowering it by about 0.0011 $OD_{570} \pm 0.0002$ (standard errors). Similarly, cysteine addition affected adherence to plastic ($\chi2(1) = 9.4344$, $p=0.00213$), lowering it by 0.0013 OD570 ± 0.0002 (standard errors). In all the strains, significant biofilm reduction was observed at the sub MIC value of 5 mM arginine and 5 mM cysteine.

Cell growth at different arginine and cysteine concentrations.

Considering that the observed decrease in biofilm formation could be the consequence of reduced cell growth, total cell populations were determined (by OD600) following the addition of 0, 2, 5, 10, and 20 mM cysteine and arginine. Contrary to what was observed for biofilm formation, the two amino acids had different effects (**Figure 4**). While the addition of cysteine significantly affected cell growth ($\chi2(1)= 11.177$, $p=0.0008$), lowering it by about 0.013 $OD_{600} \pm 0.002$ (standard errors), arginine had no significant effect ($\chi2(1)= 1.3783$, $p=0.2404$), when considering the cumulative responses of the three strains. In more details, increasing arginine concentration from 0mM to 5mM significantly ($p<0.05$) induced A9 and V80 growth while it reduced that of M23. Further addition of arginine had no significant effect on yeast growth.

FLO11 transcription in presence of 5 mM arginine or 5 mM cysteine.

FLO11, a key gene required for cell adherence to plastic surfaces and other related phenotypes, is regulated by the availability of nitrogen sources in the medium. Thus, the observed reduced biofilm

formation in the presence of arginine and cysteine could be due to the inhibition of *FLO11* transcription. The results of PCR analysis show that *FLO11* transcription is significantly reduced ($p<0.05$) following the addition of 5 mM arginine or 5 mM cysteine in all of the strains tested, with the exception of V80 in presence of cysteine. In particular, arginine addition reduced *FLO11* transcription in A9, M23, and V80 by 4.094 ± 0.06, 7.00 ± 0.07, and 1.868 ± 0.07 Log_2 (ΔΔCt), respectively. Cysteine addition reduced *FLO11* transcription in A9, M23, and V80 by 1.99 ± 0.05, 2.38 ± 0.06, and 0.31 ± 0.07 Log_2 (ΔΔCt), respectively.

Phenotype microarray (PM) analysis in the presence of 5 mM arginine or 5 mM cysteine

PM technology measures a cell's metabolic output, allowing the evaluation of cell "vitality", defined as the cell's overall physiological capabilities. Similar to the effects observed on cell growth, the addition of arginine induced a strong increase in cell metabolism (detected as an increase in the intensity of cellular respiration). An increase also was observed in response to cysteine, albeit significantly lower (**Figure S1**). To evaluate in more detail the interaction among nitrogen sources, PM respiration curves were parametrized using the spline-fitting method and the maximum respiration rates obtained were analyzed by cluster analysis (**figure 5**). Despite their distinct genetic backgrounds, all strains behaved similarly in the presence/absence of 5mM arginine or 5mM cysteine. In particular, arginine addition increased the respiration rates of all three strains in all the media tested (cluster A). The addition of cysteine (cluster C) caused very little change in the respiration rates of the three strains compared with control conditions (cluster B). Regarding possible interactions between the nitrogen sources tested and the addition of cysteine or arginine, three major clusters were identified. Cluster "1" groups the less preferred nitrogen sources, as they are poorly utilized in the control media and following cysteine addition. The nitrogen sources listed in cluster "3" induced high cellular respiration rates independently from the addition of arginine or cysteine. Finally, nitrogen sources that elicited different responses in the three conditions tested are grouped into cluster "2". In-depth analysis of the raw kinetics of the three strains in this last cluster allowed us to evaluate better the effect of cysteine addition on yeast metabolic profiles (**Figure 6**). The addition of 5mM cysteine induced lower respiration rates in media containing proline, threonine, or dipeptides containing alanine, glutamate or glycine. This suggests a negative interaction between cysteine and these nitrogen sources.

Confirmatory analyses of the effect of 5 mM arginine and 5 mM cysteine on the ∑1278b *strain*

To confirm the results obtained on the wild-type strains, the same set of analyses were carried out using *S. cerevisiae* ∑1278b that has been widely used for the study of *FLO11*-dependent phenotypes. The addition of either cysteine or arginine significantly reduced biofilm formation even

at the lowest concentration tested (**Figure S2**). ∑1278b growth was not affected by arginine addition, as also observed in the wild-type strains, but it was significantly inhibited in a dose-dependent manner in the presence of increasing concentrations of cysteine. The results of the gene expression analysis are also in accordance with the previous observation that the addition of 5 mM arginine or 5 mM cysteine causes a significant ($p<0.05$) decrease in *FLO11* transcription levels. In particular, arginine and cysteine addition reduced *FLO11* transcription by 2.874 ± 0.05 and 1.495 ± 0.06 Log_2 (ΔΔCt), respectively. Finally, PM analysis confirmed that arginine addition increased the respiration rate in all the media tested, whereas cysteine did not alter the metabolic profile of ∑1278b (**Figure S3**).

DISCUSSION

Nitrogen metabolism in *S. cerevisiae* governs the outcome of major developmental decisions that lead to cell morphological differentiation. In particular, it has been shown that limiting access to ammonia induces yeast cells to undergo pseudohyphal differentiation, invasive growth, and biofilm formation (Gagiano et al., 2002; Zara et al., 2010). The aim of the present work was to further our understanding of the role of nitrogen sources on yeast morphological differentiation. We found that the addition of 10mM histidine, arginine, lysine, cysteine, tryptophan, phenylalanine and threonine reduces biofilm formation in three *Saccharomyces cerevisiae* wild-type strains as well as in the reference strain ∑1278b. The resulting impairment of biofilm development could be due to specific or unspecific effects exerted by amino acids on yeast metabolism. Specific effects may entail the inhibition of genes or biochemical pathways required for biofilm development, whereas unspecific effects may be the consequence of reduced cell viability and vitality. Indeed, although amino acids are essential for a yeast cell's metabolism, they can also be toxic when present at high levels. Risinger et al. (2006) showed that *S. cerevisiae* cells experience severe growth defects when high concentrations of individual amino acids are added to the medium. Ruiz et al. (2017) reported a significant reduction in the biomass of *S. cerevisiae* BY4709 following the addition of 5 mM of individual amino acids to the culture media. In agreement with these studies, we observed a significant reduction in the growth of yeast cells cultured in rich media (YEPD) following the addition of histidine, arginine, lysine or cysteine at concentrations above 10mM. On the contrary, we did not observe any growth inhibition for any tested concentrations of serine, leucine, phenylalanine, proline, threonine, valine, and tryptophan.

In acknowledgment of the rising demand for the development of novel approaches to prevent the growth of microbial biofilms via, for example, the utilization of small molecules with inhibiting and

dispersing properties (López-García et al. 2002; Bou Zeidan et al. 2013), we further evaluated the effect of arginine and cysteine on biofilm formation, cell viability, vitality, and *FLO11* gene expression. Arginine and cysteine were confirmed to be the amino acids most active against biofilm formation, reducing biofilm formation by 68.6 ± 15.2 % and 54.78 ± 17.57 %, respectively. However, the results obtained suggest that the different yeast strains respond in different ways to these two amino acids.

Increasing concentrations of arginine completely abolished biofilm formation without affecting cell viability and vitality. This result is not totally in line with that of Ruiz et al. (2017), who found 5mM of arginine to significantly reduce growth, but not abolish it. These authors assessed the effect of amino acids on the BY4709 strain, which has a laboratory yeast S288c background; whereas we used three wild-type strains isolated from a winery environment. Considering that wine is particularly rich in arginine, usually as high as 3 mM (Bouloumpasi et al. 2002), wine strains of *S. cerevisia*e could be better adapted to high concentrations of this amino acid in the medium. In addition, PM analysis showed that arginine significantly reduced the lag phase, suggesting that arginine was preferred among the nitrogen sources tested. The same conclusion was drawn by Jiranek et al. (1995), who assessed the order of nitrogen source preference for S. *cerevisiae* growing on synthetic grape juice media. Furthermore, Crepìn et al. (2012) found that wild strains of *S. cerevisiae* removed arginine at high rates from a chemically defined medium.

Previous studies have shown a correlation between the assimilation of specific nitrogen sources and *FLO11*-dependent phenotypes. Lorentz and Heiman (1998) showed that the ammonium transporter Mep2p is essential for *FLO11* transcription and the filamentous growth of *S. cerevisiae*. Torbensen et al. (2012) suggested that amino acid transporters are similarly essential for *FLO11* transcription. In this respect, assimilation of nitrogen sources is regulated in yeast by the nitrogen catabolite repression (NCR) mechanism, which controls the general amino acid permease Gap1p and the ammonium permease Mep2p. Gap1p is proposed to be the major yeast transporter of arginine under arginine-rich conditions (Crepìn et al. 2012). In addition, Torbensen et al. (2012) found that Gap1p induces invasive growth by increasing amino acid pool levels and eliciting *FLO11* gene expression. Considering these facts, we hypothesized that arginine affects biofilm formation through a direct mechanism, mediated by the Gap1p transporter, that includes the regulation of *FLO11* transcription. Indeed, we found that the transcription level of *FLO11* was significantly lower in the presence of arginine compared with that for control medium for all the strains tested.

The activity of cysteine against bacterial and yeast biofilms has already been described. Zhao and Liu (2010) showed N-acetyl cysteine to exert anti-bacterial properties towards *Pseudomonas*

aeruginosa and proposed that it may mediate the detachment of *P. aeruginosa* biofilms. Moreover, L-cysteine, mixed with other amino acids and nisin, prevents biofilm formation by *Streptococcus mutans* (Tong et al. 2014). With regard to yeasts, Abd El-Baky et al. (2014) found that N-acetyl cysteine inhibits and eradicates *Candida albicans* biofilms.

Our results support the hypothesis that cysteine counteracts biofilm formation through an indirect mechanism. Indeed, both cell viability and cell vitality were significantly affected by cysteine addition. The toxicity of cysteine against yeast cells, for concentrations above 20 mg/L, has been known for over 50 years (Maw, 1963). Pinu et al. (2014) suggested that *S. cerevisiae* only consumes very small quantities of cysteine as a source of sulfur and not in order to support growth. Thus, the reduced *FLO11* transcription levels observed after cysteine addition could be related to the general impairment of cell metabolism.

In conclusion, L-arginine and L-cysteine are promising molecules for the prevention of biofilm formation by yeast. Regarding their mode of action, we found that antimicrobial and anti-biofilm activities were not necessarily linked in all the different strains investigated. Arginine addition increases both cell viability and respiration rates, whereas the addition of cysteine causes a reduction in both of these parameters. Their use should therefore be assessed in function of the action required towards cell growth.

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Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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FIGURE LEGENDS

Fig 1 Dose-response curves for *S. cerevisiae* strains treated with different amino acids. Curves show means and standard deviations for three replicates of OD600 measurements 48 h post inoculation in YPD. The *S. cerevisiae* strains investigated were A9 (gray circles), M23 (black squares), and V80 (gray triangles)

Fig 2 Adhesion ability of *S. cerevisiae* strains in the presence of 10 mM of single amino acids. Adhesion ability is expressed as a percentage with respect to the adhesion values (A_{570}) measured in the control condition (SC medium without amino acid). The *S. cerevisiae* strains represented are A9 (panel A), M23 (panel B), and V80 (Panel C). Data are means ± standard deviations of five independent measurements

Fig 3 Adhesion ability of *S. cerevisiae* after 48 h incubation in the presence of increasing concentrations of arginine (panel A) or cysteine (panel B). Each panel is subdivided into three sub-panels showing the effect of 0, 2, 5, 10, and 20 mM of the specified amino acid on each of the strains tested, namely A9, M23, and V80. Box-Plots indicate the minimum, first quartile, median, third quartile, and maximum absorbance values (A_{570})

Fig 4 Cell density of *S. cerevisiae* after 48 h incubation in the presence of increasing concentrations of arginine (panel A) and cysteine (panel B). Each panel is subdivided into three sub-panels showing the effect of 0, 2, 5, 10, and 20 mM of the specified amino acid on each of the strains tested, namely A9, M23 and V80. Box-Plots indicate minimum, first quartile, median, third quartile, and maximum absorbance values (A_{600})

Fig 5 Cluster analysis of PM data. Maximum AWCD values were obtained from each well of the following: PM3B plate (control), PM3B plate + 5mM arginine (Arg), and PM3B plate + cysteine (Cys). Metabolic outputs were acquired from the *S. cerevisiae* strains A9, M23, and V80. Data are the means of two independent biological replicates

Fig 6 Metabolic outputs of yeast cells in response to 5 mM cysteine in wells A01, B09, B11, H03, H04, and H10 of the PM3B plate. Black circles: 5mM cysteine; gray triangles: control. Data show mean AWCD from *S. cerevisiae* strains A9, M23, and V80 raw values

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strains	Genetic background	Reference
A9	Wild flor strains of *S. cerevisiae* isolated from Arvisonadu wine	Budroni et al. 2000
M23	Wild flor strains of *S. cerevisiae* isolated from Malvasia wine	Zara et al. 2009
V80	Wild flor strains of *S. cerevisiae* isolated from Vernaccia wine	Zara et al. 2009
Σ1278b	Wild-type; closely related to the reference strain S288c	ATCC 42800. Brandriss and Magasanik, 1979

Table 2. qRT-PCR primer pairs used in this study

Target	Forward primer	Reverse Primer
ACT1	5’-CGTTCCAATTTACGCTGGTT-3’	5’-TCAGCAGTGGTGGAGAAAGA-3’
ARF1	5'-AGATCGCGTATTGGTGAAGC -3'	5'-CATGGCTTCTGGCAAATCTT -3'
SUM1	5'-TTGTGGAACCATCAACGAAA -3'	5'-TTCTTTGCGGTACGGAAGTC -3'
FLO11	5'-AGGTTCAAATGGTGCCAAGA-3’	5'-AGCCACGCTAGAAGCAGAAG-3'

Figure 1

HISTIDINE

ARGININE

LYSINE

CYSTEINE

SERINE

LEUCINE

PHENYLALANINE

PROLINE

THREONINE

VALINE

TRYPTOPHANE

A9

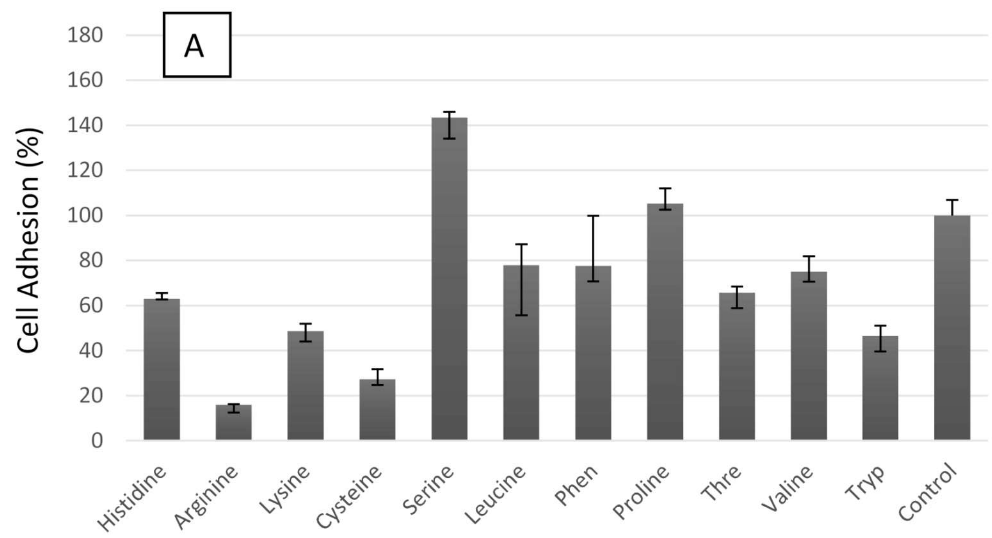
M23

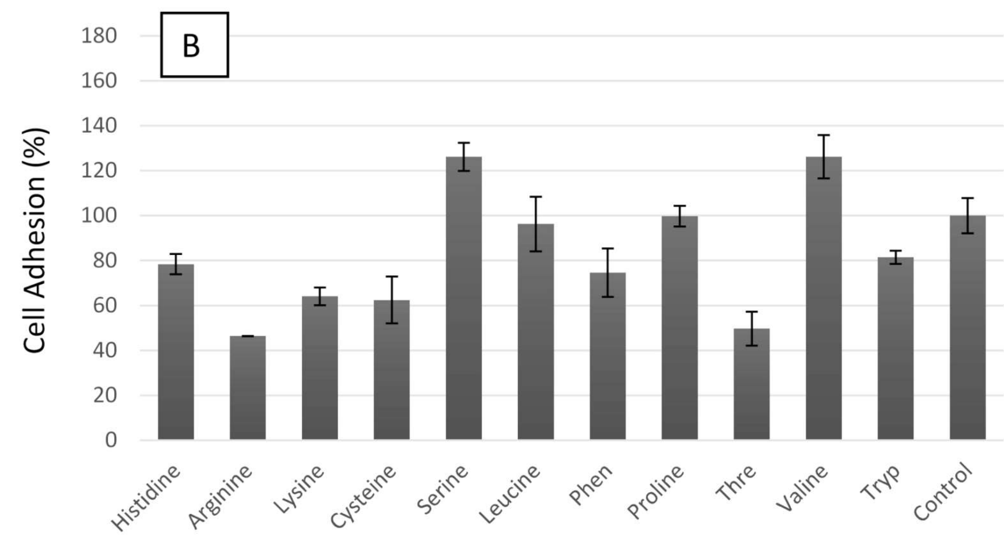
V80

Cell concentration (A_{600})

Aminoacid concentrations (mM)

Figure 2





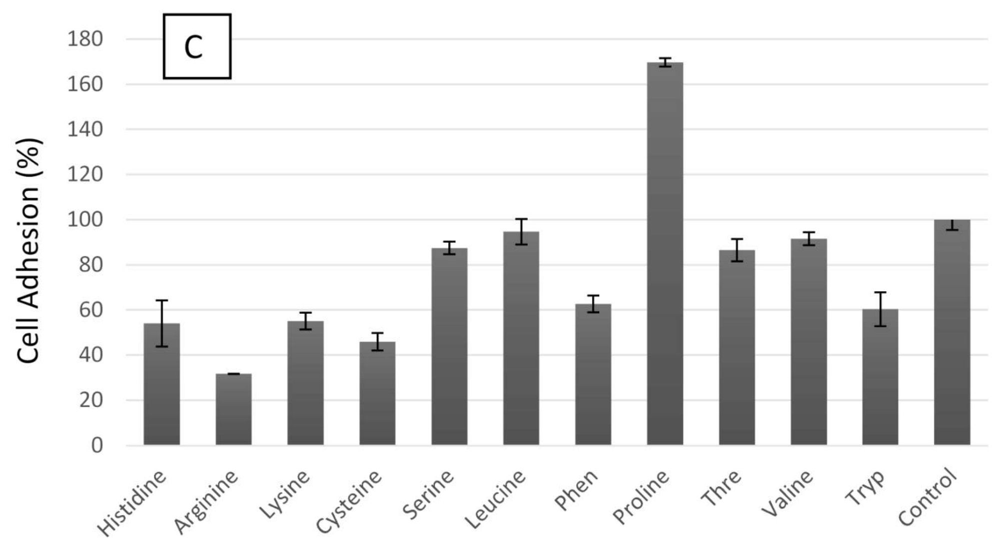


Figure 3

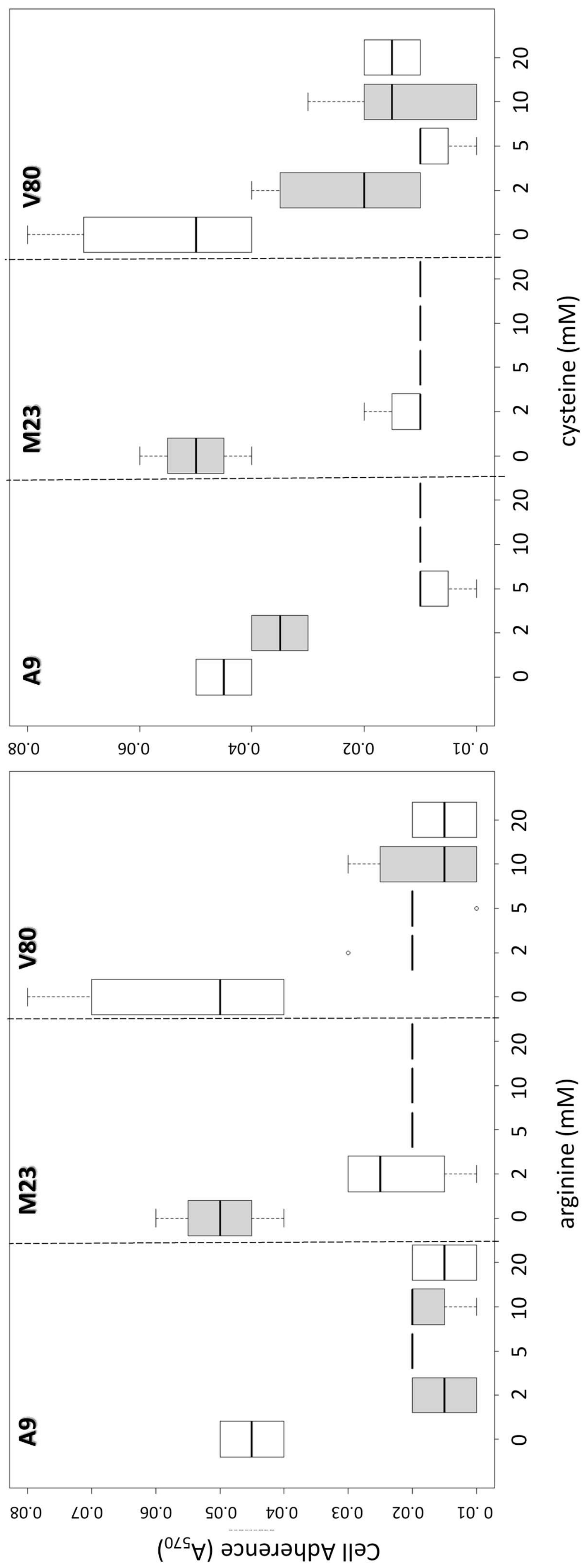
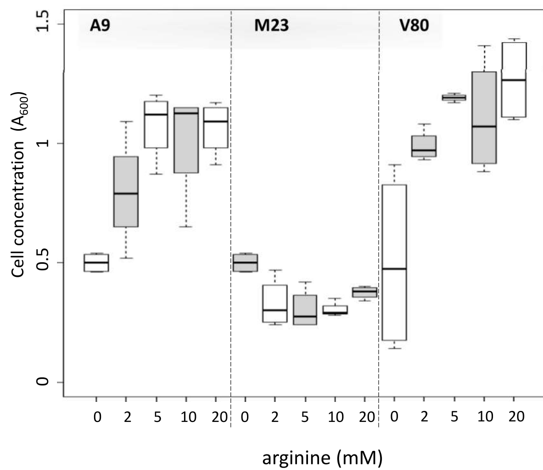


Figure 4



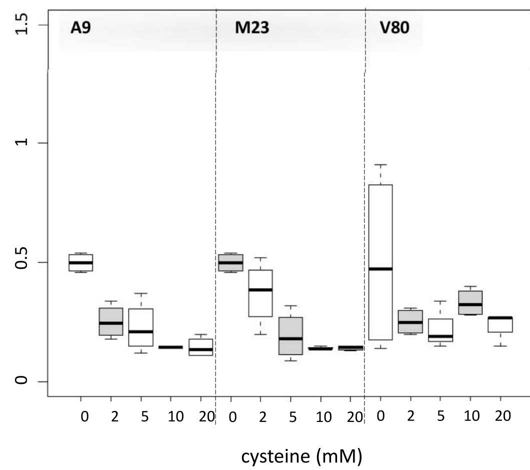


Figure 5

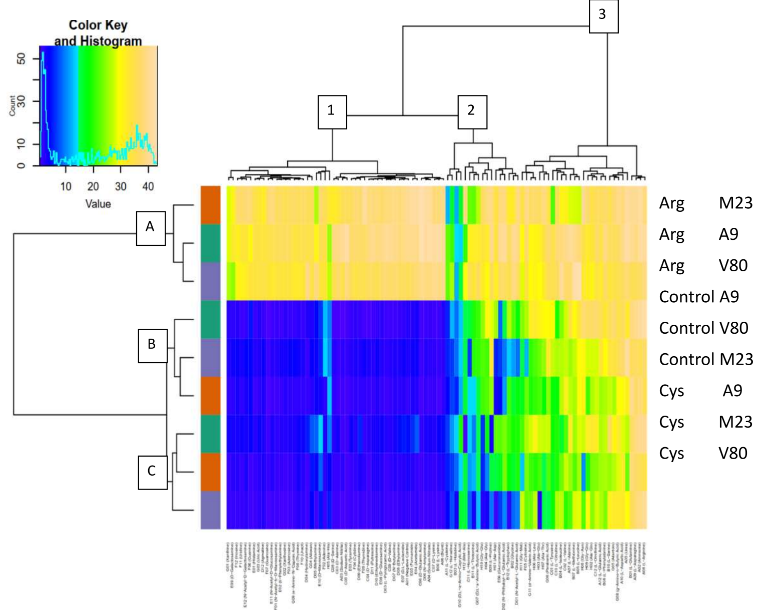


Figure 6