Exploratory study on the occurrence and dynamics of yeast-mediated nicotinamide riboside production in craft beers

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Abstract

Several health benefits are related to the administration of nicotinamide riboside (NR), a form of Vitamin B3, and its precursors nicotinamide mononucleotide (NMN) and NAD^+. Therefore, considerable interest is currently devoted to the potential therapeutic value of their supplementation, thus justifying scientific studies on the distribution of these molecules in foods and beverages. In this study, the three vitamers were quantitatively analyzed in ten craft beers for the first time. All beers from different commercial *S. cerevisiae* strains contained NAD^+. NR, NMN and NAD^+ were mostly present in beers produced with *Saccharomyces cerevisiae* strain US-05. Interestingly, the three vitamers were not detectable in beers produced with a commercial strain of *Saccharomyces pastorianus*. Data from laboratory-scale beer production using *S. cerevisiae* strain US-05 showed that the addition of hops during the fermentation process significantly increased NR production. The rapid increase in NR formation only occurred if both hops and yeast were present, and the burst was also confirmed in fermentations trials performed with *S. cerevisiae* strain $CBS1171^T$ and by replacing wort with YPD medium. The experimental model proposed in the present study can serve as baseline for further research aimed at investigating the yeast-hop interaction at metabolic and molecular levels. In addition to highlighting the potentialities of microorganisms to act as biological factories for beneficial molecules to humans, these findings open new intriguing perspectives for the development of innovative fermented foods naturally enriched in NR and its precursors.

Keywords: Vitamin B3, NR, NMN, NAD^+, *Saccharomyces cerevisiae*, hop, beer, yeast and hop synergy

1. Introduction

Vitamin B3, which is also called Niacin or vitamin PP (Pellagra-Preventing), includes nicotinic acid (NA), its amide nicotinamide (Nam), and nicotinamide riboside (NR), the last discovered form (Bieganowski & Brenner, 2004). Once taken with the diet, NA, Nam and NR are transported inside the cells where they can be transformed into NAD^+, which represents the biologically active form of vitamin B3. NAD^+ itself is present in food together with the phosphorylated form of NR, i.e., nicotinamide mononucleotide (NMN). In the human gut, both dietary NAD^+ and NMN can be transformed into the three forms of the vitamin by a combined action of enzymes of the intestinal mucosa and microbiota (Bogan & Brenner, 2008).

Numerous lines of evidence indicate that administration of the vitamin NR and its precursor NMN to mice at doses ranging from 100 to 500 mg/kg/day causes a significant increase in the intracellular content of NAD^+ in many tissues and organs, which is reflected in improvements in energy metabolism and mitochondrial function. As a result, supplementation of NR or NMN shows both preventive and therapeutic properties in neurodegenerative diseases (e.g., Parkinson's and Alzheimer's), metabolic syndrome (Hartnup's disease), human immunodeficiency virus (HIV), autoimmune diseases, alcohol dependence, anorexia and diseases related to aging that seem to reproduce the symptoms of pellagra (Chi & Sauve, 2013; Hong, Mo, Zhang, Huang, Wei, & 2020; Rajman, Chwalek, Sinclair, & 2018; Ruggieri, Orsomando, Sorci, & Raffaelli 2015; Yoshino, Baur, & Imai, 2018). Doses yielding health benefits in mice models are much higher than the amounts of NR and NMN that have been documented to date in a common balanced diet. NMN has been detected in many natural foods, such as broccoli, tomatoes, mushrooms, cabbage, shrimp, avocado and beef meat, with a maximum concentration of 1.88 mg/100 g (Mills et al., 2016), whereas NR has only been documented at micromolar concentrations in milk to date (Trammell, Yu, Redpath, Migaud, & Brenner, 2016; Ummarino et al., 2017). Considering the beneficial effects attributed to these molecules, it is important to extensively investigate their distribution in food. Beside the natural

presence of NR in milk, it has been hypothesized that microbial metabolic activities could contribute to its occurrence in fermented food and beverages (Chi & Sauve, 2013). Indeed, different authors reported the ability of *Saccharomyces cerevisiae* to actively secrete this vitamin (Bogan et al., 2009; Lu, Kato, & Lin, 2009). Such evidence prompted us to investigate the presence of NR and its dietary precursors, NMN and NAD^+, in craft beer. Beer is a beverage consumed worldwide that is derived from a biochemical process based on the fermentation of sugary substrates present in the wort beer by the action of yeast (Anderson, Santos, Hildebrand, & Schug, 2019; Nardini & Garaguso, 2020). This process is an alcoholic fermentation that leads to the production of ethanol, carbon dioxide and other secondary compounds, such as polyphenols particularly phenolic acids (benzoic and cinnamic acid derivatives) and flavonoids, important for the characterization of the product (Nardini & Garaguso, 2020). The raw materials necessary for the beer production include water, barley (*Hordeum vulgare*) and other cereals eventually used, hops (*Humulus lupulus*), and yeast. Yeast strains used for the brewing process belong to the genus *Saccharomyces* spp. Traditionally, these yeast strains are classified as yeast for low fermentation, namely, *Saccharomyces pastorianus* (operating temperature 8-15°C), and yeast for high fermentation, namely, *S. cerevisiae* (operating temperature 15-23°C). The use of *S. cerevisiae* cultures (top yeast) produces a high fermentation beer (top fermentation) called Ale, in which the yeasts tend to rise to the surface positioning in the foam. In contrast, *S. pastorianus* (bottom yeast) produces low fermentation (bottom fermentation) in which the yeast at the end of the fermentation process are found on the bottom of the beer based on their ability to flocculate (Lager beer) (Iserentant, 2003; Lodolo, Kock, Axcell, & Brooks, 2008; Speers, Tung, Durance, & Stewart, 1992; Verstrepen, Derdelinckx, Verachtert, & Delvaux, 2003). The brewing process can be divided in four different main phases: malting (transformation of barley into malt), mashing (production of wort), fermentation by yeast (transformation of sugars in ethanol, carbon dioxide and secondary compounds) and downstream processes (maturing, bottling, and packaging) (Anderson et al., 2019). At the end of maturation (generally 3-4 weeks), the beer must be subjected to filtration processes to separate the suspended solids and to pasteurization to produce a

more stable final product. In a few cases, there is another step of hop addition to the beer. This step, which is called dry hopping, can be performed before fermentation, at the end of fermentation, or during a second fermentation in the bottle. Craft beers, unlike industrial beers, are usually subjected to a second fermentation process in the bottle, by the addition of sugars and yeast. The beer produced in craft breweries differs from industrial beers also because they are consumed unfiltered and unpasteurized (Garofalo et al., 2015). Moreover, craft breweries produce mainly Ale beers, so they utilize predominantly *S. cerevisiae* strains (Iattici, Catallo, & Solieri, 2020).

In this work, levels of NR and its precursors NMN and NAD^+ have been quantified in different craft beers via an enzyme-coupled assay (Ummarino et al., 2017). In addition, laboratory-scale fermentations have been established using different *S. cerevisiae* strains added to wort or YPD medium to shed light on the mechanism of NR production.

2. Material and methods

2.1. Craft beer and wort sampling

Ten craft beers of different brewing styles were analyzed for the presence of NR, NMN and NAD^+. In particular, two samples from different batches of each beer type were collected from two craft breweries located in the Marche region (Central Italy).

All the worts used to produce these beers were collected at the end of boiling step and stored at 4°C. Table 1 summarizes the yeast species and strains, ingredients and alcohol percentage (%) of the craft beers under study, and Figure 1 shows a flow diagram of their manufacturing process.

2.2. Fermentation trials

The yeast strains *S. cerevisiae* US-05 (Fermentis Lessafre Italia, Parma, Italy) and *S. cerevisiae* CBS 1171^T (from the *Centraalbureau voor Schimmelcultures*, Filamentous fungi and Yeast Collection, The Netherlands) were grown on Yeast Extract Peptone D-glucose (YPD) (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L) medium at 25°C for 72 hours. Yeast strains were inoculated in sterile flasks containing 100 mL of wort (or 200 mL YPD) to reach a final concentration of approximately 6 $\log_{10}$ cfu/mL. In a conventional fermentation trial, after 9 days at 21°C, two different hops were added in pellet form (dry hopping). These hops consisted of amarillo (alpha acid: 9.0%) (4 g/L) and centennial (alpha acid: 8.5%) (4 g/L) varieties. After this addition, the maturation continued at 4°C. At the 16^{th} day of fermentation, dextrose (7 g/L) was added; it was dissolved in 600 µL of sterile water by heating at 100°C for 5 minutes. The brewing process continued at 4°C until the 45^{th} day unless otherwise stated.

At different days during the fermentation, aliquots of samples were removed to analyze the following: i) the yeast concentration through viable counting on YPD agar (agar 18 g/L) following decimal serial dilutions on sterile peptone water (peptone 1 g/L); ii) the content of NR, NMN and NAD^+ as described in paragraph 2.3.

2.3 NR, NMN and NAD^+ quantitation

Samples of beers and aliquots from the fermentation trials were subjected to acid-soluble nucleotides extraction. To this end, 0.5 mL were centrifuged at 16000 x g for 5 minutes at room temperature before adding 250 µL of 1.2 M $HClO_4$. After 15 minutes at 4°C, samples were centrifuged as described above and 700 µL of the supernatants were added to 170 µL of 1.0 M K_2CO_3 to reach a pH value of approximately 7.0. Neutralized samples were centrifuged again, and the supernatants were used for the quantitation of NR, NMN and NAD^+ through the enzyme-coupled assay described by Ummarino et al. (2017). Briefly, the coupled assay consists of two consecutive reactions catalyzed by recombinant bacterial NR kinase and recombinant murine NMN adenylyltransferase that

stoichiometrically convert NR to NMN and NMN to NAD^+, respectively. The produced NAD^+ is then quantified by the fluorometric cycling assay described by Zamporlini et al. (2014).

2.4. Statistical Analysis

NR, NMN and NAD^+ data are represented by boxplot that represent the interquartile range (IQR) between the first and third quartiles, and the line inside the plot represents the median (2nd quartile). Data were subjected to one-way ANOVA to examine the development across time. When significant differences were found, Duncan's multiple range test was used. Linear regression model was used to reveal the associations between NR, NMN and NAD^+ as a function of time. A P-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 21.0 and R software.

3. Results and Discussion

3.1 NAD^+, NMN, NR determination in craft beers

Ten different types of craft beers were analyzed for the presence of NR, NMN and NAD^+ as described in paragraph 2.3. All the beers analyzed were prepared with *S. cerevisiae*, except beer B1, which was produced by *S. pastorianus.* The results of the screening are shown in Figure 2. To the best of the authors knowledge, this is the first report showing the presence of NAD^+, NMN and NR in beer. In more detail, NAD^+ was detected in all the studied beers with the exception of B1. Its content showed marked variability, even within different batches of the same beer. Levels ranged from 1.10 nmol/mL to 17.80 nmol/mL and were intriguingly very similar to those determined in ovine and caprine milk (Ummarino et al., 2017). The highest amount of NAD^+ ($P<0.05$) was present in the two beers produced using the *S. cerevisiae* strain S-04. In particular the highest value was reached in sample

B6 followed by sample B5 while lowest amount and minimal differences were observed among the others (Figure 2). These results suggest that S-04 has the capacity among the tested strains to produce and release the highest amount of NAD^+ during the brewing process. In addition, the only beer lacking NAD^+ was the one prepared using *S. pastorianus* (sample B1), thus indicating that NAD^+ production could be species specific. Interestingly, in this latter beer, NR and NMN were also undetectable (Figure 2). Notably, among the beers prepared with *S. cerevisiae*, all the beers produced with the strain US-05 contained NR. Three of the beers also contained NMN (sample B7, B9 and B10), although at levels generally lower than NR and without differences in the quantity among them (Figure 2, $P>0.05$). NR concentrations ranged from 0.48 to 3.25 nmol/mL, and these values were unexpectedly very similar to those determined in bovine milk (from 0.5 to 3.6 nmol/mL) (Trammell et al., 2016; Ummarino et al., 2017). In particular, NR was mostly detected in samples B9 and B10. However, no difference in concentration between the two was observed (Figure 2, $P>0.05$). Furthermore, samples B7 and B8 showed comparable quantities of NR but always significantly lower than B9 and B10 (Figure 2, $P<0.05$). In contrast to NR, NMN was not always present in both tested batches of beer, and when present, its content was approximately 0.9 nmol/mL. This content closely resembles that measured in ovine and donkey milk, where NMN ranges from 0 to 1.0 nmol/mL (Ummarino et al., 2017). In general, NMN levels in beer and milk are lower than those measured in other foods, such as tomato, avocado and beef meat (from 0.78 nmol/mg to 4.79 nmol/mg) (Mills et al., 2016).

All the worts were negative for the presence of NR and its metabolic precursors (data not shown).

The data obtained in the present study clearly indicate that the production of the three metabolites is a typical feature of the *S. cerevisiae* species and is strain dependent.

205 *3.2 NR, NMN and NAD^+ determination in lab-scale produced beer*

206

207 Screening of NR, NMN and NAD^+ in the different craft beers indicated that only the beers produced
208 with *S. cerevisiae* strain US-05 contained all the three vitamers (Figure 2). Prompted by these results,
209 a replicate of the beer B9 on a laboratory scale (B9L) was established to monitor the production of
210 the molecules during the entire fermentation process. The B9 sample was chosen based on the
211 availability of the corresponding wort by the brewery. The fermentation was performed as described
212 in paragraph 2.2, and the production of the three metabolites as well as the viable yeast counts were
213 monitored during the entire process (Figure 3A).
214 On the 2^{nd} day of fermentation, the concentration of the yeast strain significantly increased from the
215 starting inoculum ($P < 0.05$) reached the value of 7.5 $\log_{10}$ cfu/mL and then remained constant until
216 the end of the process ($P < 0.05$). Different fluctuations in the levels of the metabolites were recorded.
217 After an initial lag of approximately 2 days, the amount of NR significantly increased, exhibiting a
218 burst after the addition of the hop, i.e., after the 9^{th} day. In fact, the value of NR shifted from 1.3
219 nmol/mL on the 9^{th} day to 3.4 nmol/mL on the 14^{th} day, representing an approximately 3-fold
220 increase. The linear regression model showed a significant increase in the production of NR across
221 time (Adjusted R-squared: 0.9694, p-value: < 0.05). The addition of sugar after 16^{th} days did not
222 affect the trend of NR. The trend for NAD^+ was very different from that of NR. In more detail, in the
223 first two days of the process, this metabolite significantly increased to approximately 3 nmol/mL
224 ($P<0.05$) and remained at this level until the addition of the hops. After the 9^{th} day, it sharply
225 decreased to very low levels (Adjusted R-squared: 0.2701, p-value< 0.05). Regarding NMN, after a
226 sharp increase in the first two days to a value similar to that of NAD^+, it remained constant until the
227 23^{rd} day and then started to slowly decrease (Adjusted R-squared: 0.003437, p-value: 0.2992).
228 A comparison of the levels of the molecules of interest in the B9 beer (Figure 2) and in the lab-scale
229 beer B9L on the last day of the fermentation (Figure 3A) revealed that the final amount of NR in B9L

was considerably increased compared with that in B9 beer, whereas NAD^+ was lower. On the other hand, NMN levels were very similar.

The differences in NR and NAD^+ content between B9 and B9L samples might be due to several reasons. In fact, although we used the same ingredients at the same concentrations as in the brewery, the yeast strain used by the brewery was in a lyophilized form (not grown on YPD for 72 hours), and the second fermentation occurred in a closed bottle in the brewery. Furthermore, the time elapsed from bottling to sampling was unknown.

The results in Figure 3A suggest that the addition of the hops might be responsible for boosting NR and decreasing NAD^+ during the fermentation trial. This behavior was confirmed by the linear regression model where a positive relationship among NAD^+ and NR was observed (Adjusted R-squared: 0.3632, p-value < 0.05).

To better define the role of hops in the change in metabolites levels, a control fermentation trial without the addition of hops was established (Figure 3B). After the initial increase to approximately 3 nmol/mL, NAD^+ continued to slightly increase throughout the fermentation process (Adjusted R-squared: 0.6937, p-value < 0.05), whereas NMN remained constant (Adjusted R-squared: 0.1112, p-value < 0.05). NR showed a very slight increase from the 8^{th} day until the end of the fermentation (Adjusted R-squared: 0.7835, p-value< 0.05). Altogether, these results suggest that the addition of hops stimulates NR production and induces degradation of both NAD^+ and NMN. However, the relationship was verified by the linear model only by considering the behavior of NR and NAD^+ (Adjusted R-squared: 0.56, p-value< 0.05).

S. cerevisiae cells constitutively produce, release and import NR (Bogan et al., 2009; Lu et al., 2009), whereas no information is available on the ability of plant cells to release NR. It is therefore tempting to hypothesize that hops might enhance the yeast's ability to produce and release the vitamin. In this view, yeast cells would facilitate NAD^+ supply to hop cells by providing the NR precursor. Metabolic interaction between different cell-types through the exchange of extracellular metabolites is a well-known mechanism, and evidence has been provided that different cell types might support each

256 other's NAD^+ pools by providing NR as NAD^+ precursor (Kulikova et al., 2015). Morover, it is
257 interesting to note that Steyer, Tristam, Clayeux, Heitz, & Laugel (2017) highlighted a synergy
258 between several yeast strains and hop varieties on beer volatile compounds production, thus
259 indicating that an interaction between hop compounds and yeast metabolism exists although it
260 remains to be investigated. Unfortunately, the lack of information on the regulation of intracellular
261 NR generation and release does not allow to explain the mechanism underlying the metabolic
262 interaction between yeast and hop cells. Furthermore, to the authors' knowledge, data regarding NR,
263 NMN and NAD^+ dynamics during a craft beer production is lacking in the scientific literature, thereby
264 preventing further comparison.
265
266 *3.3 Effect of wort and S. cerevisiae strain on the production of NR, NMN and NAD^+*
267
268 The present study also investigated whether the presence of the wort was required for the NR bursting
269 effect exerted by the hops. To this end, a fermentation trial substituting the wort with YPD medium
270 and determining the concentrations of the molecules of interest on different days was performed. As
271 shown in Figure 4A, the change in metabolites levels during the process closely resembled that
272 observed in the fermentation of wort. Indeed, in YPD, the addition of hops caused a rapid increase in
273 NR (Adjusted R-squared: 0.866, p-value < 0.05) and a decrease in NAD^+ (Adjusted R-squared:
274 0.1699, p-value < 0.05) thus indicating that the presence of wort is not essential for the production of
275 the vitamin. Even in YPD a linear trend was observed by the consumption of NAD^+ and the
276 production of NR (Adjusted R-squared: 0.2964, p-value < 0.05). Dextrose was not added in these
277 fermentation trials since it was previously demonstrated that it did not influence the vitamer trends.
278 It was therefore asked whether the presence of the yeast was required for the effect of the hops and
279 whether the effect was strain specific. Figure 4B shows that NR and NAD^+ are not produced in the
280 absence of yeast. On the other hand, a slight amount of NMN was produced when hops were added
281 to the YPD medium (Figure 4B). The amount was approximately three fold lower than that measured

in the presence of the *S. cerevisiae* strain US-05 and decreased to undetectable levels at the end of the process.

Figure 4C shows the levels of the metabolites during the fermentation of YPD inoculated with *S. cerevisiae* strain CBS 1171^T. The tendency of NR to increase following the addition of hops was observed also in the presence of this strain. Strain CBS 1171^T released NR on the 4^{th} day, whereas the vitamin was detectable on the 9^{th} day with the strain US-05 (Adjusted R-squared: 0.9138, p-value < 0.05). Moreover, in the absence of hops, NR decreased in the strain US-05 under sustained fermentation, whereas it continued to slowly increase when strain CBS 1171^T was present (Adjusted R-squared: 0.6907, p-value < 0.05). NMN production was different in the two fermentations. The strain US-05 released NMN from the beginning of the process, and the addition of hops did not change its concentration up to the 15^{th} day. Then, a progressive decrease was noted (Adjusted R-squared: 0.04175, p-value: 0.2346). On the other hand, strain CBS 1171^T produced NMN only after the addition of hops, and the levels slowly decreased during the fermentation (Adjusted R-squared: 0.3007, p-value < 0.05). The trend of NAD^+ production was similar in the two fermentations. In fact, the addition of hops also caused a decrease in NAD^+ levels with the CBS 1171^T strain. CBS 1171^T released more NAD^+ than US-05.

Altogether, these results indicated that both hops and yeast are required during the brewing process to increase NR production. Moreover, the NR bursting effect seems to be *S. cerevisiae* strain and wort independent. Further studies to elucidate the interaction between hops and *S. cerevisiae* cells at intracellular metabolic level could shed more light on the NR bursting effect exerted by hops.

4. Conclusion

In the present work, for the first time, the presence of NR and its dietary precursors NMN and NAD^+ in craft beers was quantitatively assessed, suggesting potential beneficial properties of such a low alcoholic beverage. The presence of the three vitamers in the beers under study was *S. cerevisiae*

strain-dependent. Overall, all craft beers prepared with different *S. cerevisiae* strains contained NAD^+, further highlighting the potentialities of microorganisms to act as biological factories for beneficial molecules to humans. By reproducing a lab-scale fermentation process either in wort and YPD medium, a significant increase in NR levels was observed after the addition of hops, and both the yeast *S. cerevisiae* and hops are required for such a burst to occur, thus indicating that a yeast and hops sinergy on NR production occurs. The present study represents the first attempt to provide an experimental model to study the hop-yeast interaction at metabolic and molecular levels. Finally, these findings open new intriguing perspectives for the development of innovative fermented foods naturally enriched in NR and its precursors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors would like to thank the breweries “Birrificio SanGermano18” and “Birrificio del Gomito” and their owners (Corrado Pirani and Paolo Ragni, respectively) for their voluntary help in the supply of yeast strains, beer and wort samples and information about brewing production. This work was partly supported by PRIN2017CBNCYT to NR.

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FIGURE CAPTIONS

Figure 1. Flow diagram of manufacture of the craft beers B1-B8 (A); Flow diagram of manufacture of the craft beers B9 and B10 (B).

Figure 2. Boxplots showing the levels NR, NMN, NAD^+ concentrations (nmol/mL) detected in the different craft beers under study.

Different letters at the base of the boxes indicate significant differences for each metabolite among beers ($P < 0.05$).

Figure 3. NR, NMN and NAD^+ concentrations in B9L (A) and control (B) during the fermentation process.

Measurements were performed in duplicate and the means ± standard deviation were reported.

Figure 4. Effect of hop addition on NR, NMN and NAD^+ levels in YPD medium inoculated with *S. cerevisiae* strain US-05 (A), without yeast inoculation (B), and inoculated with *S. cerevisiae* strain CBS 1171^T (C).

Hop was added at the 9^{th} day. Measurements were performed in duplicate and the means ± standard deviation were reported.

Table 1. *Saccharomyces* yeast strains, ingredients and alcohol percentage (%) in craft beer samples

Sample	Yeast species	Strain	Ingredients	% alcohol
B1	*Saccharomyces pastorianus*	W34/70	H_2O, hop, yeast, sugar, barley and wheat malt	6.0
B2	*Saccharomyces cerevisiae*	S-33	H_2O, hop, yeast, sugar, barley malt	5.5
B3	*Saccharomyces cerevisiae*	WB-06	H_2O, hop, yeast, sugar, barley and wheat malt, wheat	6.3
B4	*Saccharomyces cerevisiae*	WB-06	H_2O, hop, yeast, sugar, barley and wheat malt	5.8
B5	*Saccharomyces cerevisiae*	S-04	H_2O, hop, yeast, sugar, barley malt	6.3
B6	*Saccharomyces cerevisiae*	S-04	H_2O, hop, yeast, sugar, barley and wheat malt	6.6
B7	*Saccharomyces cerevisiae*	US-05	H_2O, hop, yeast, sugar, barley and wheat malt	6.6
B8	*Saccharomyces cerevisiae*	US-05	H_2O, hop, yeast, sugar, barley malt	5.4
B9	*Saccharomyces cerevisiae*	US-05	H_2O, hop, yeast, sugar, barley malt, oat flakes	5.5
B10	*Saccharomyces cerevisiae*	US-05	H_2O, hop, yeast, sugar, barley and wheat malt, oat flakes	5.5

Fig. 1

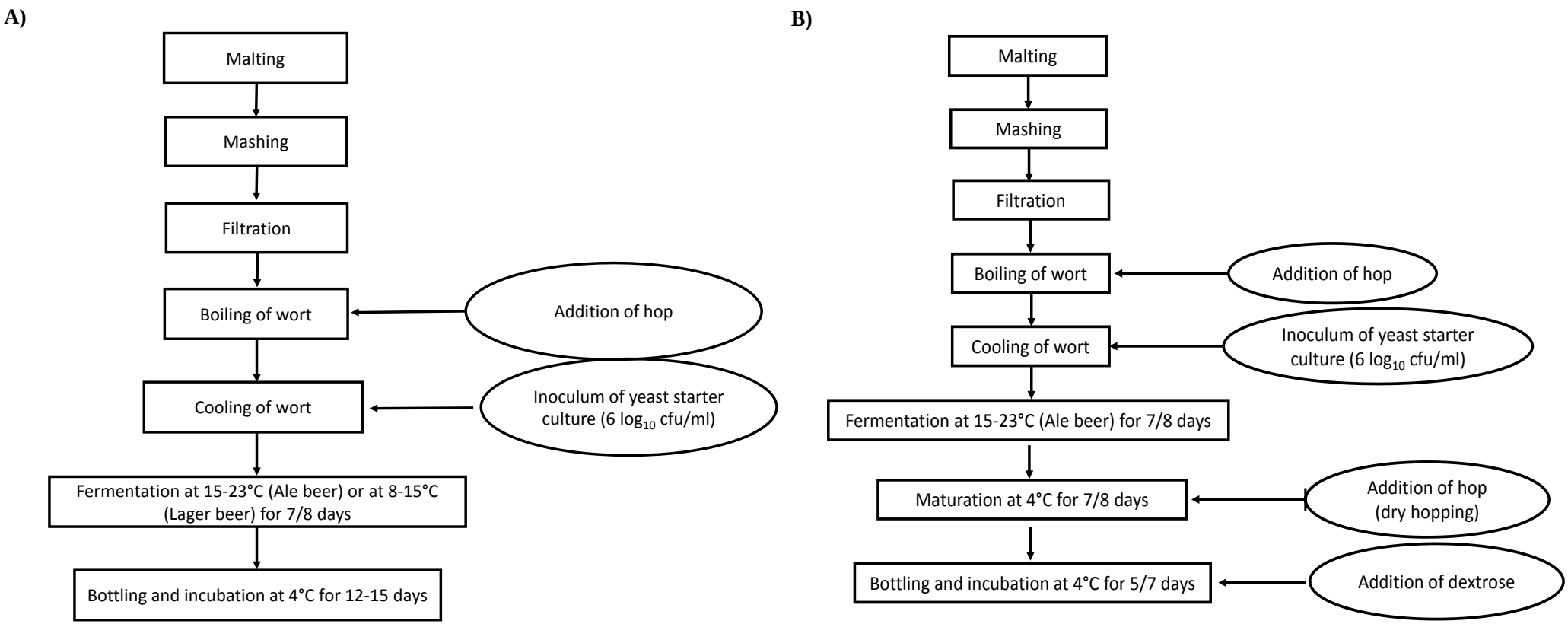

Fig. 2.

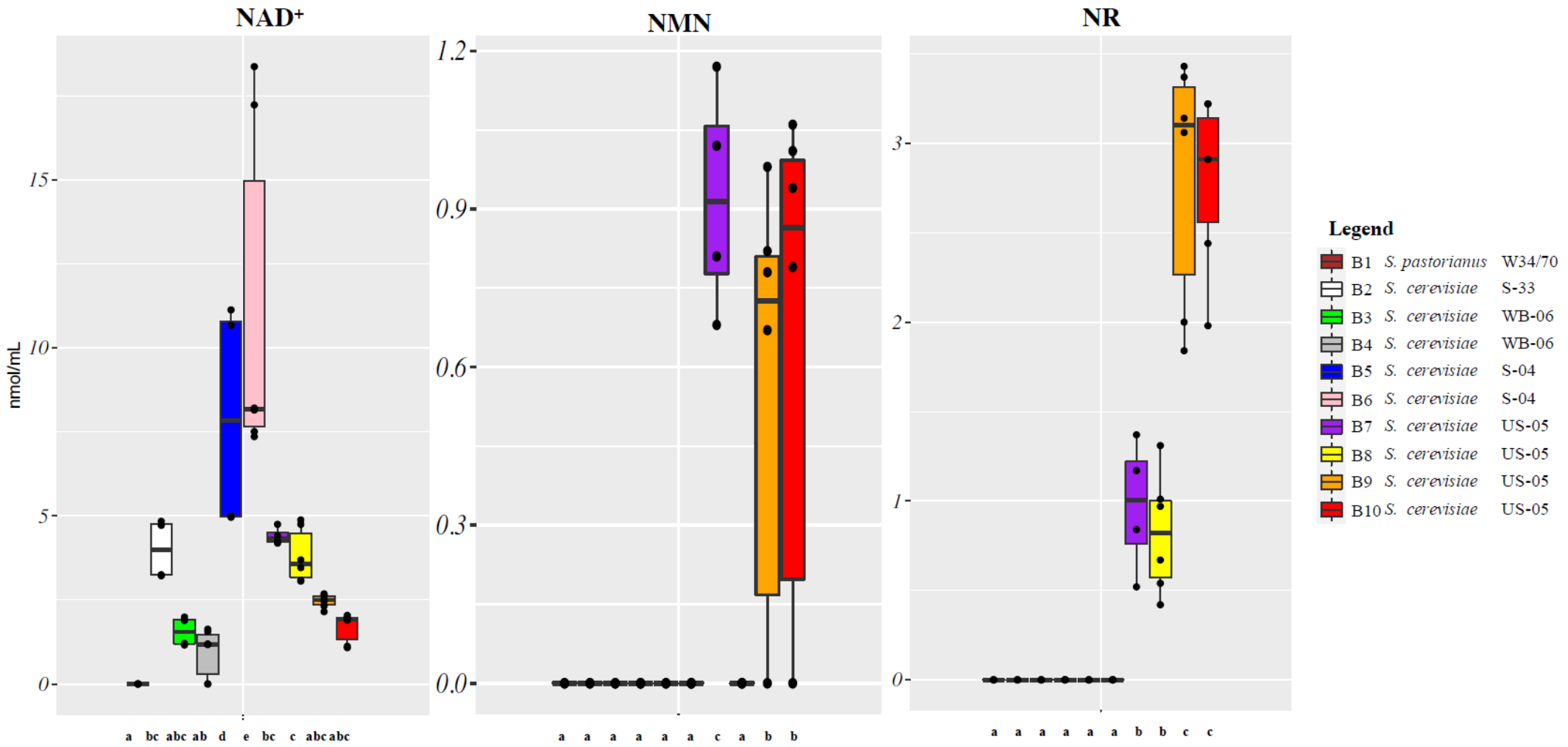

Fig. 3

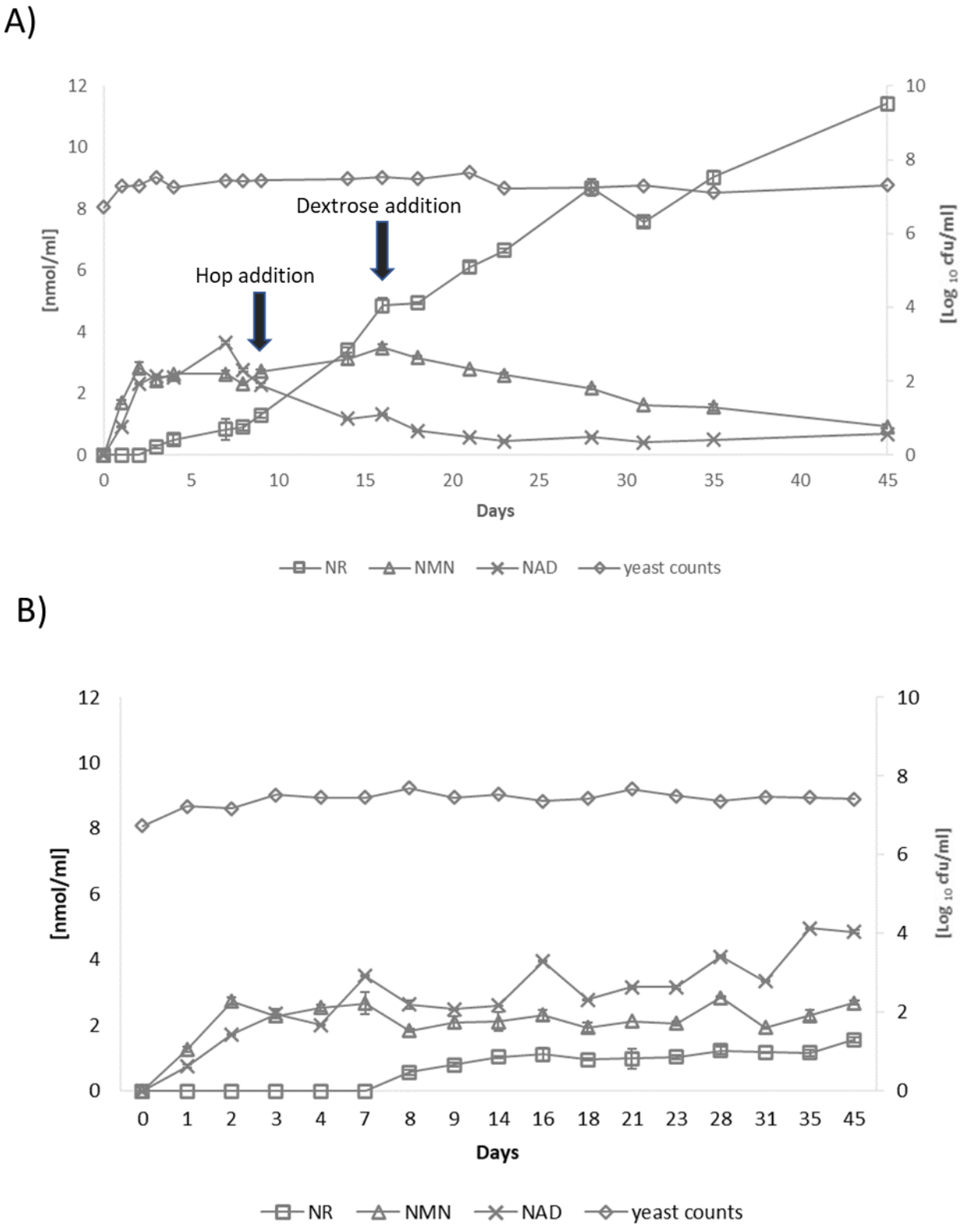

Fig. 4