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Nicotinamide mononucleotide (NMN) deamidation and indirect regulation of the NAD metabolome

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ABSTRACT

Treatment with nicotinamide mononucleotide (NMN) is a prominent strategy to address the age-related decline in nicotinamide adenine dinucleotide (NAD⁺) levels for maintaining aspects of late-life health. It is assumed that exogenous NMN is directly incorporated into the NAD⁺ metabolome in mammals via the canonical recycling pathway. Here, we show that NMN can undergo direct deamidation and incorporation via the de novo pathway, which is in part mediated by the gut microbiome. Surprisingly, isotope labelling studies revealed that exogenous NMN treatment potently increased the endogenous production of unlabelled NAD metabolites, suggesting that exogenous NMN impacts the NAD metabolome through indirect means, rather than through its direct incorporation. This included a striking increase in endogenous production of the metabolites nicotinic acid riboside (NaR) and nicotinamide riboside (NR) which was amplified in antibiotics treated animals, suggesting the production of endogenous NaR/NR through altered metabolic flux, enzyme kinetics and/or an as-yet unidentified pathway that interacts with the gut microbiome.
Nicotinamide adenine dinucleotide (NAD⁺) is an essential redox cofactor central to metabolic processes such as glycolysis, the tricarboxylic (TCA) cycle and fatty acid oxidation. NAD⁺ is also consumed by enzymes such as the sirtuins and poly(ADP-ribose) polymerase (PARP) enzymes which are mediators of genome stability and DNA repair. Given the essential role of this metabolite, the decline in NAD⁺ that occurs during biological ageing and disease states has gained attention as a target for therapeutic intervention. Strategies to boost NAD⁺ levels through supplementation with NAD precursors such as nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) are emerging as promising therapeutics. Historically, dietary supplementation with the NAD precursors nicotinic acid (Na) or nicotinamide (Nam) was used to prevent chronic NAD deficiency, which causes pellagra. When these micronutrients are replete, the step converting Nam into NMN by the enzyme nicotinamide phosphoribosyltransferase (NAMPT) is rate limiting in NAD synthesis, and the use of NAD precursors that occur after this step, namely NMN and NR, have gained prominence as a strategy to raise NAD⁺.

One surprising aspect of this strategy is the striking appearance of the deamidated metabolite nicotinic acid adenine dinucleotide (NaAD) following oral delivery with the amidated metabolite NR. NR is phosphorylated into NMN by NR kinases (NRK1/2), and then adenylated into NAD⁺ by NMNAT enzymes (NMNAT1-3), effectively bypassing NaAD, which is an intermediate of the Preiss-Handler or de novo pathway. In contrast, bacteria have a well-characterised NMN deamidase enzyme, PncC that prevents the accumulation of NMN, which inhibits the bacterial DNA ligase. One theory to explain the increase in NaAD with NR treatment could be that NMN and NR assimilation follows a non-canonical route that combines steps of both microbial and mammalian processes, whereby NMN is deamidated into NaMN or NaR prior to its uptake into mammalian tissue, and then assimilated into NAD⁺ via the intermediate step of NaAD. This could explain the appearance of NaAD following NR supplementation, however an unexplained aspect is that the delivery of labelled NR results in the formation of unlabelled NaAD.
Here, we use targeted metabolomics to trace the *in vitro* and *in vivo* metabolism of strategically designed NMN isotopologues to answer these questions. We show that NMN can be incorporated following its deamidation and metabolism via the *de novo* route, which is in part mediated by the microbiome. We further show that ablation of the microbiome by antibiotic treatment increases the uptake and conversion of orally delivered NMN into the NAD metabolome, and that isotope labelled NMN overwhelmingly presents in intestinal tissue in the form of NaR and NR. Contrary to the assumption that exogenous NMN treatment raises NAD⁺ levels solely through its direct incorporation into the NAD metabolome, we show that treatment with isotope labelled NMN increases the levels of endogenous, unlabelled NAD metabolites. Overall, our results provide unique insights into the assimilation of orally delivered, exogenous NMN into gastrointestinal tissue, and raise questions around how exogenous precursors alter the NAD metabolome.

**RESULTS**

**NMN treatment alters the *de novo* arm of NAD⁺ synthesis**

According to canonical models of mammalian NAD homeostasis, the metabolism of NMN, an amidated intermediate in the recycling pathway, does not intersect with the *de novo* pathway, which utilises deamidated intermediates. Unlike mammals, bacteria present in the gut microbiome do encode deamidase enzymes such as PncC, which deamidates NMN into nicotinic acid mononucleotide (NaMN) for metabolism via the *de novo* pathway. To test whether the gut microbiome alters the *in vivo* metabolism of orally administered NMN, we used mice that were exposed to a course of antibiotics to ablate the gut microbiome (Supp. Fig. 1). These animals received a bolus of NMN (500 mg/kg) by oral gavage, and four hours later, animals were sacrificed and tissues rapidly preserved for targeted metabolomic analysis (Fig. 1). We focused our analyses on the gastrointestinal tract (GIT) and the liver, as these two tissues have high levels of NAD synthetase (NADS) activity, and are the primary sites of uptake and metabolism for orally delivered
compounds. In agreement with previous work\textsuperscript{25}, NMN treatment increased the abundance of the de-amidated metabolites NaR and NaMN in both the gastrointestinal tract (GIT) (Fig. 1a, b) and liver (Fig. 1d, e), while NaAD was increased in the liver (Fig. 1f), matching previous findings for NR\textsuperscript{25}. Interestingly, this was completely abolished in antibiotic treated animals, where NMN treatment instead led to a spike in the amidated metabolites NR (Fig. 1g, j) and NMN (Fig. 1h, k), and abolished the increase in liver NaAD (Fig. 1f). To highlight the inverse relationship between amidated and de-amidated metabolites during antibiotics treatment, the abundance of each deamidated metabolite was expressed as a ratio of its amidated counterpart (Fig. 1m-r), highlighting a profound role for the microbiome in dictating the roles of the de-amidated and amidated arms of NAD metabolism.

**Strategic isotope tracing of NMN metabolism**

We next sought to carefully test whether exogenous NMN was indeed undergoing direct deamidation prior to its incorporation into the NAD metabolome using isotope tracing studies. We designed two separate isotopologues of NMN that were strategically labelled at positions that would answer our hypothesis of NMN deamidation. The first of these, designated as \textbf{NMN1}, was $^{13}$C labelled at all five carbon positions of the ribose moiety for an M+5 mass shift, and $^{15}$N labelled at the pyridine ring for an overall M+6 mass shift (Fig. 2a, Supp. Fig. 2). In the last step of the \textit{de novo} pathway, the enzyme NAD synthetase (NADS) amidates the carboxylic acid of NaAD using an ammonia intermediate derived from the amide group of glutamine, yielding glutamate (Fig. 2). By delivering the \textbf{NMN1} (M+6) isotope in the presence of $^{15}$N-amide labelled glutamine (M+1), the presence of M+7 labelled NAD$^+$ with an additional mass shift from the nicotinyl amide would indicate that the original amide N atom had been lost during deamidation and replaced by the $^{15}$N amide from $^{15}$N-Gln, indicating incorporation of NMN into NAD$^+$ via prior deamidation and the \textit{de novo} pathway (Fig. 2a). To complement this experiment, we designed a second isotope, designated as \textbf{NMN2}, where all five carbons of the ribose moiety were $^{13}$C labelled, and both the pyridine ring and primary amide positions were $^{15}$N labelled, for an overall M+7 mass shift (Fig. 2b, Supp. Fig. 2). When delivered in a separate
experiment, if NMN2 (M+7) underwent deamidation prior to its incorporation, the $^{15}$N amide would be lost and replaced by an unlabelled amide from the endogenous glutamine pool, resulting in M+6 labelled NAD$^+$. By comparing the ratios of M+7 and M+6 labelled NAD$^+$ in each experiment, we could quantify the proportion of NMN that had been incorporated into NAD$^+$ following deamidation and assimilation by the *de novo* pathway. This would be supported by comparing the ratios of M+1 and M+2 labelled Nam, which is released by NAD$^+$ consuming enzymes, however this interpretation would be complicated by the recently described role of the bacterial nicotinamide (Nam) deamidase PncA in systemic mammalian NAD$^+$ homeostasis$^{41}$. By using triple-quad mass spectrometry and multiple reaction monitoring (MRM) for targeted metabolomics, we could further refine these data to determine where mass shifts occurred, including whether Nam was labelled at the pyridine ring or amide positions, and whether M+6 or M+7 labelling of NAD was from the NMN rather than the adenosine phosphate moiety.

$^{15}$N-Gln labelling of NAD$^+$ biosynthesis

To test whether this scheme would lead to labelling of the NAD pool as anticipated, we first used primary rat hepatocytes grown *in vitro*, to avoid contributions from the microbiome. Hepatocytes were treated for 24 hr with $^{15}$N-glutamine (M+1) in the presence or absence of NMN1 (M+6), or with NMN2 (M+7) (Fig. 3). Cell lysates were subject to targeted metabolomic analysis to assess the degree of isotope incorporation into each metabolite (Fig. 3a-e). Delivery of each of these isotopes yielded the expected M+6 and M+7 mass shifts of NMN (Fig. 3b) as well as its de-phosphorylated counterpart NR (Fig. 3a), which is consistent with the indirect transport of NMN$^{26}$, though these data do not exclude the direct transport of NMN via the putative transporter SLC12A8$^{42}$ – for this reason, the data in this investigation could be interpreted as evidence for deamidation of NMN and/or NR, rather than NMN alone. To test the strategy of using $^{15}$N glutamine to label NAD$^+$ synthesis, we compared the ratio of M+1 (nicotinamide labelled) to M+0 (endogenous) NAD$^+$ (Fig. 3f). As expected, $^{15}$N-Gln treatment increased M+1 labelling of endogenous NAD$^+$, with M+1 NAD$^+$ labelling in untreated
samples due to baseline levels of naturally occurring isotopes. High levels of M+1 NAD\(^+\) labelling (Fig. 3f) were observed in samples treated with **NMN1**, likely due to recycling of the M+1 labelled Nam moiety (Fig. 3g) following the breakdown of NAD\(^+\) (Fig. 2), or by cleavage of the NMN glycosidic bond between the ribose and nicotinamide groups. As expected, treatment with **NMN1** (M+6) and **NMN2** (M+7) led to M+6 and M+7 labelling of NAD\(^+\) (Fig. 3c). While we had hypothesised that *in vivo* treatment with **NMN1** (M+6) and \(^{15}\)N-Gln (M+1) would lead to M+7 labelled NAD\(^+\) due to the deamidation of NMN by the gut microbiome, in these primary hepatocytes we observed that \(^{15}\)N-Gln co-treatment with **NMN1** (M+6) increased the formation of M+7 labelled NAD\(^+\), when compared to **NMN1** (M+6) alone (Fig. 3h). In line with the expected recycling of labelled Nam from NAD\(^+\) (Fig. 2a), this increased formation of M+7 labelled NAD\(^+\) during **NMN1** (M+6) and \(^{15}\)N-Gln co-treatment was matched by an identical increase in M+2 labelling of free Nam (Fig. 3i), which was re-incorporated into the nicotinyl moiety of NAD\(^+\) (Fig. 3j). \(^{15}\)N-Gln treatment increased M+1 labelling at the amide position of Nam (Fig. 3i), but not the base N atom of the pyridine ring (\(\text{Nam}_{\text{base}}\), Fig. 3k), which does not undergo substitution by NADS, with **NMN1** (Fig. 2a) treatment serving as a positive control for labelling at this position. Overall, these data verified our system of labelling, and demonstrated the specificity of our targeted analytical approach, based on triple quadrupole mass spectrometry and MRM targeted metabolomics. As mammals do not encode a known NMN, NR or Nam deamidase enzyme, we next sought to measure the incorporation of labelled NMN into the NAD\(^+\) metabolome of bacteria, which can deamidate NAD precursors including NMN\(^{36}\) and Nam\(^{43}\).

**NMN deamidation by bacteria**

Unlike mammals, bacteria rely on an NAD\(^+\) dependent DNA ligase that is inhibited by NMN, the product of its own reaction\(^{37-39}\), resulting in the accumulation of intracellular NMN during exponential growth\(^{44}\). This NMN is salvaged through the bacterial NMN deamidase PncC, yielding NaMN as a substrate for NAD\(^+\) synthesis by the Preiss-Handler pathway\(^{36}\). To model whether
extracellular NMN would undergo deamidation by bacteria, growth phase *E. coli* cultures were supplemented with **NMN1** (M+6) (Fig. 2a) and subjected to targeted metabolomics of both cell lysates and extracellular culture media (Fig. 4). Consistent with the role of PncC in NMN metabolism in bacteria, treatment with labelled NMN resulted in the rapid incorporation of isotope labels into NaMN, with vastly increased labelling of NaMN compared to NMN (Fig. 4). Similarly, a role for the Nam deamidase PncA is strikingly reflected in the abundance of nicotinic acid (Na) compared to nicotinamide (Nam) in the cell pellet compared to the culture supernatant, where the ratio of Nam to Na in growth media was completely reversed. Overall, the avid uptake of NMN, followed by its rapid shunting into deamidated metabolites such as NaMN (Fig. 4) supported our hypothesis that the gut microbiome could contribute to the metabolism of orally administered NAD precursors such as NMN.

**Antibiotic treatment alters NMN deamidation in vivo**

To directly trace whether the increase in deamidated metabolites following NMN administration (Fig. 1) was indeed due to the direct deamidation and incorporation of these metabolites, we next delivered our strategically designed isotopes into animals that had similarly been treated with antibiotics to deplete the gut microbiome, as confirmed by reductions in faecal DNA concentration (Supp. Fig. 1a), full-length 16S rRNA sequencing (Supp. Fig. 1c) and reduced alpha diversity (Supp. Fig. 1e-r). Following antibiotic treatment, animals received a single oral gavage (50 mg/kg) of the **NMN1** (M+6) isotope (Fig. 2a), in parallel with an i.p. bolus of **15**N-Gln (M+1). Four hours later, animals were sacrificed and tissues rapidly preserved for targeted metabolomic analysis (Fig. 5, Supp. Fig. 3-4). In a separate experiment, a different cohort of antibiotic treated animals (Supp. Fig. 1b, d, f-r) received a bolus of the **NMN2** (M+7) isotope (Fig. 2b) alone, following which tissues were similarly collected 4 hr later for targeted metabolomic analysis (Fig. 6, Supp. Fig. 5, 6).

In tissues from animals treated with **NMN1** (M+6), the deamidation of NMN could be quantified by comparing the ratio of M+6 NAD⁺, which would assume incorporation following the canonical route,
to M+7 NAD⁺, which had incorporated an extra mass shift from co-treatment with ¹⁵N-Gln (M+1) (Fig. 2a, 7a). In this experiment, an increased ratio of M+7 to M+6 labelled NAD⁺ would indicate the deamidation of NMN. The reason for using ratios, rather than the overall amounts of each isotope (Fig. 5, Supp. Fig. 3, 4), is that they internally control for differences in bioavailability within each animal. From the intact labelling of NAD⁺ in the GIT from NMN₁ treatment, around 13% was M+7 labelled (Fig. 7b, c). Importantly, these data likely underestimates incorporation via the deamidated route, as this scheme relied on the availability of exogenous ¹⁵N-Gln relative to the endogenous pool of unlabelled Gln, which composed only 9-13% of the total plasma Gln pool at the 4 hr timepoint (Fig. 7k). Consistent with our hypothesis, the ratio of M+7 to M+6 labelling in the GIT was reduced in antibiotic treated animals (Fig. 7b), suggesting reduced deamidation of orally administered NMN when contributions from the microbiome were reduced. This was reflected by a reduction in the ratio of M+2 to M+1_base labelled Nam in the GIT, liver and plasma (Fig. 7d-f), however this change also likely reflected reduced contributions from the bacterial nicotinamide de-amidase PncA⁴³ following antibiotic treatment⁴¹. This change in Nam labelling carried into the M+2 to M+1 ratio of NMN (Fig. 7g, h) and NAD⁺ (Fig. 7i, j), with reduced labelling ratios of these recycled isotopes during antibiotic treatment likely reflecting a combination of possible NMN/NR deamidation (Fig. 7a, b), and contributions from the bacterial Nam deamidase PncA⁴¹.

To complement this approach, in the NMN₂ (M+7) experiment (Fig. 2b), we would anticipate that deamidation by the microbiome would result in loss of the ¹⁵N amide label, resulting in the formation of M+6 NAD⁺ at the expense of M+7 NAD⁺ (Fig. 2b, 7l). In contrast to the previous NMN₁ experiment, the ratio of M+7 to M+6 NAD⁺ would instead decrease as the rate of deamidation increased. Further, interpretation of deamidation in this NMN₂ experiment was not limited by the availability of exogenous ¹⁵N-Gln relative to a large, endogenous pool of Gln, as was the case with the NMN₁ experiment (Fig. 7a, k). In this experiment, the ratio of M+7 to M+6 NAD⁺ was around 3:1 (Fig. 7m, n), suggesting that around 25% of orally administered NMN undergoes deamidation.
prior to its intact incorporation into NAD⁺. In agreement with the previous experiment, the ratio of M+7 to M+6 labelled NAD⁺ was increased in the GIT and liver of antibiotic treated animals (Fig. 7m, n). This was similarly matched by an increased ratio of M+2 to M+1base labelled Nam in the GIT (Fig. 7o), liver (Fig. 7p) and plasma (Supp. Fig. 7q) of antibiotic treated animals, reflecting decreased incorporation following deamidation, though this could instead be due the deamidation of Nam rather than NMN. As in the previous experiment, these labels were recycled into M+2 labelled NMN (Fig. 7r, s) and NAD⁺ (Fig. 7t, u). In addition to differences in the isotope labelling of NAD⁺ (Fig. 7), these experiments replicated the inverse relationship between NaMN and NMN levels following antibiotic treatment (Fig. 5b, e, Fig. 6b, e) observed in our earlier experiment with unlabelled NMN (Fig. 1b, h, n). Overall, these data from two complementary isotope labelling approaches support the concept that orally delivered NMN or NR can undergo deamidation prior to incorporation, and a role for the microbiome in mediating this. While these data could in part explain the spike in the de-amidated metabolites NaMN and NaAD following treatment with the amidated precursors NR25 or NMN (Fig. 1), it is important to note that when measured as a proportion of the overall NAD⁺ pool, the contribution of both M+7 and M+6 intact labelled NAD⁺ was small. Partially labelled NAD⁺ (M+2) was around 10-fold more abundant than intact labelled NAD⁺ (M+7) (Fig. 5d, 6d, Supp. Fig. 3-6), indicating either cleavage of the labile glycosidic bond of NMN, or rapid recycling of NAD⁺45. Following cleavage of the glycosidic bond to release free Nam, its deamidation in the GIT46,47 by the bacterial enzyme PncA41 also likely contributes to these changes.

Exogenous NMN boosts the endogenous NAD⁺ metabolome

The abundance of partially labelled NAD⁺ with labelling at the Nam position only is consistent with previous findings45 that orally delivered NMN and NR undergo cleavage at the glycosidic bond to release free Nam, with only a small proportion of orally delivered material being incorporated into tissues intact. Given that NMN and NR are overwhelmingly incorporated in the form of free Nam45, why do the downstream biological effects of NR or NMN differ from the delivery of Nam25,48-50, a
widely available nutrient present in dietary sources? One basic assumption is that exogenous NAD precursors increase levels of NAD$^+$ and other metabolites due to their direct incorporation, as expected by classic mass-balance models. Following the delivery of near 100% isotope labelled material, we found that the increase in metabolites such as NR includes the increased production of endogenous, unlabelled metabolites. For example, treatment with NMN1 or NMN2 in antibiotics treated animals increased levels of unlabelled NaR (Fig. 5f, 6f, Supp. Fig. 3f, 5f) in the GIT, and increased unlabelled NR (Fig. 5b, 5p, 6b, 6p Supp. Fig. 3b, 5b) and NaAD (Fig. 5n, 6n, Supp. Fig. 4g, 6g) in the liver. Notably, treatment with labelled NMN in antibiotics treated animals increased the production of unlabelled NR in the GIT by over 3-fold, where this single metabolite accounted for the vast majority of the entire NAD metabolome in the gut, as summarised in Figure 5p and 6p.

**Host-microbe interactions in the bioavailability of orally delivered NAD$^+$ precursors**

Another unexpected aspect of these data was the overall increase in levels of these metabolites as a result of antibiotics treatment alone, which more than doubled the labelling of the metabolites NMN, NR, NAD$^+$ and Nam (Fig. 1g-i, 5a-d, 6a-d, Supp. Fig. 3h-k, 4h-k, 5h-k, 6h-k). This increase even occurred in unlabelled metabolites in animals that did not receive exogenous NMN (Supp. Fig. 3a-d, 5a-d). When NMN1 (M+6) was delivered, the incorporation of exogenous labels into NAD$^+$ metabolites was vastly increased in antibiotic treated animals, in the case of NR in the gut, by an order of magnitude (Fig. 5c, 5p, Supp. Fig. 3i), a trend that was recapitulated in a separate cohort of animals receiving the NMN2 (M+7) isotope (Fig. 6b, 6p, Supp. Fig. 5i) and in animals that received unlabelled NMN (Fig. 1g). The overwhelming abundance of NR as the dominant NAD metabolite in the GIT, especially following NMN delivery in antibiotics treated animals is worthy of later investigation, as the abundance of this single metabolite was greater than all other NAD$^+$ metabolites combined, including NAD$^+$ itself (summarised in Fig. 5p, 6p). Together, the striking increase in the uptake and overall abundance of both labelled and unlabelled NAD metabolites in antibiotics treated animals suggests that the microbiome could be in competition with mammalian tissue for the uptake
of orally administered, exogenous NAD precursors, and the uptake of NAD precursors from dietary sources. Future studies should measure isotope labelling of NAD⁺ metabolites in faecal contents of mice to confirm whether these compounds are being utilised by the microbiome, rather than being excreted via other mechanisms, and should use animals in which the microbiome has been reconstituted to control for the effects of antibiotics treatment.

**Evidence for NMN uptake following dephosphorylation into NR**

NMN uptake can occur following the dephosphorylation of NMN into NR by the cell surface enzyme CD73, prior to uptake by ENT nucleoside transporters and re-phosphorylation into NMN inside the cell by NRK1/2 (Fig. 8)²⁶,²⁷. Alternatively, the solute carrier protein SLC12A8 has been described as a dedicated NMN transporter⁴². As with CD73 and ENT, SLC12A8 is located on the apical side of the intestinal tissue. As both mechanisms could co-exist, the question is the degree to which each mechanism contributes to the uptake of NMN⁵¹. If the direct route via SLC12A8 prevailed, we would expect to see high levels of labelled (M+7 or M+6) NMN, with lesser uptake of labelled NR. In contrast, if the indirect transport of NMN following its dephosphorylation into NR was dominant, there would be a higher levels of NR labelling. In primary hepatocytes (Fig. 3), NMN1 (M+6) treatment resulted in near complete labelling of the NR pool (Fig. 2b), with slightly lesser labelling of the NMN pool (Fig. 2a). Strikingly, *in vivo* treatment showed strong labelling of the NR, but not NMN pools (Fig. 5b, c; Fig. 6b, c; Supp. Fig. 3a-b, h-i; Supp. Fig. 5a-b, h-i; Supp. Fig. 7). Intact (M+6 or M+7) labelled NR levels (Supp. Fig. 3i, 5i) were five-fold higher than endogenous NR (M+0) (Fig. 5c, 6c, Supp. Fig. 3h, 5h; Fig. 8), which increased to a ten-fold greater enrichment with antibiotics treatment (Supp. Fig. 8). In stark contrast, only around 5% of the NMN pool was M+7 labelled (Fig. 5b, Fig. 6b; Supp. Fig. 3a, h; Supp. Fig. 5a, h, Fig. 8). The inability of exogenous NMN to displace the endogenous NMN pool, combined with the surge of labelled NR, suggests that NMN uptake bypasses direct transport, and would instead support the dephosphorylation of NMN into NR to facilitate its intestinal absorption (Fig. 8). If direct transport of NMN does occur, it (along with
NR) is in competition with the microbiome, as even when M+6 or M+7 labelling of NMN was observed at low levels, this only occurred in antibiotic treated animals (Fig. 5b, 6b). An important caveat of this interpretation is that limited availability of isotope labelled material meant that this study used a single time point, rather than a time course which also encompassed very early timepoints, possibly missing the minute-order kinetics of direct NMN transport that were previously reported\textsuperscript{20,52}.

**Chronic NMN treatment does not alter microbial diversity**

Given the potential role for host-microbiome interactions in the metabolism of orally delivered NMN, it might be expected that NMN treatment would alter the composition of the microbiome. To test this, we treated aged, 97-week old animals with chronic NMN dosing via addition to drinking water (~400 mg/kg/day) for 8-10 weeks prior to cull\textsuperscript{7,9,11,53}. 16S rRNA long-read sequencing revealed no overall change in the alpha diversity of the gut microbiome (Fig. 9), however there were a number of changes among individual species, available as a Supplementary File.

**DISCUSSION**

Together, this work provides evidence for the partial incorporation of exogenous NMN into the NAD metabolome via the deamidated route, and for contributions of the gut microbiome to the metabolism of exogenous NMN. This is in line with recent findings around the role of Nam deamidation by bacteria\textsuperscript{41}, however a role for the microbiome in the uptake of exogenous NAD precursors has not been described. It will be interesting to determine whether this relationship persists for other precursors, or is unique to NMN. Rather than being evidence for a “competition” relationship, differences in the uptake of exogenous NMN could reflect its role as an inhibitor of bacterial DNA ligase\textsuperscript{37-39}, and bacterial mechanisms to prevent its accumulation. In addition to NMN deamidase enzymes, this could include a role for bacterial NAD glycohydrolase enzymes, and/or bacterial SARM-like enzymes\textsuperscript{54}. 
While intravenous delivery of NR or NMN results in a small degree of intact assimilation into peripheral tissues such as the liver, kidney and muscle, oral delivery of NMN results in hepatic cleavage at the glycosidic bond yielding free nicotinamide due to the action of the liver. Our results were in close alignment with those findings, where the ratio of intact M+7 or M+6 to M+0 unlabelled NAD\(^+\) was around 2%, whereas the ratio of M+2 labelled to M+0 unlabelled NAD\(^+\), presumably as a result of incorporation of free Nam, was over 10% (Fig. 5d, k; 6d, k). Given this evidence for the decomposition of NMN into free Nam prior to its uptake, a key question is why downstream precursors in NAD\(^+\) synthesis such as NMN and NR lead to different outcomes compared to Nam alone. A surprising aspect of these results was that treatment labelled NMN led to an increase in unlabelled NAD metabolites. In the case of both NR and NaR, treatment with NMN1 (M+6) or NMN2 (M+7) led to a stark increase in endogenous (M+0) levels, particularly in antibiotics treated animals (Fig. 5c, f, 6c, f, Supp. Fig. 3b, f, 5b, f). We also observed that exogenous NMN increased liver NaAD levels (Fig. 1f, 5g, n, 6g, n), as was previously reported for exogenous NR treatment, however the majority of this increase was from unlabelled NaAD (Supp. Fig. 4g, 6g). These results were similar to those of Trammel et al, where isotope tracing of double-labelled NR showed that while total NaAD levels increased by over 40-fold following NR treatment, only around 45% of this NaAD was isotope labelled – with the endogenous origin of the remaining 55% remaining unexplained. We argue that these findings run against the assumed model that exogenous NAD\(^+\) precursors raise NAD\(^+\) levels through their direct incorporation into the NAD metabolome, and instead could suggest that treatment with exogenous precursors could indirectly trigger endogenous NAD\(^+\) biosynthesis. The mechanism for this is not yet clear, though given the profound effect of antibiotic treatment, in particular for the overwhelming abundance of NR in the gut (Fig. 5c, p, 6c, p, Supp. Fig. 3b, 5b), it is likely to involve interplay with the gut microbiome. One possibility for the changes in endogenous NAD\(^+\) metabolites following exogenous NMN/NR treatment could be that
exogenous NMN or NR treatment triggers unknown signalling pathways that indirectly alter endogenous NAD metabolism, rather than the direct incorporation of exogenous material.

Another explanation is that increased substrate levels alter the in vivo kinetics of NAD biosynthetic enzymes, increasing the utilisation of endogenous substrates. An important question regarding NR in particular is how its endogenous production is increased by exogenous NMN. NR is available from dietary sources, and is an intermediate in the uptake of extracellular NMN. NMN accumulation in neurons can trigger cell death through the NADase SARM1, and disposal of NMN through its adenylation into NAD$^+$ can protect against neuronal death. It is possible that exogenous NMN triggers pathways that degrade endogenous NMN into NR, which could act as a reservoir for NAD precursors, however CD73, the NMN ectonucleotidase that carries this out, sits on the extracellular face of the plasma membrane rather than the cytosol. In addition, the sheer molar quantity of unlabelled NR that we observed in the gut (Fig. 1g, 5c, 6c) relative to other metabolites (Fig. 5p, 6p) challenges this idea. NAD homeostasis is tightly maintained within a defined range, and the activity of NMNAT enzymes that carry out the last step of NAD biosynthesis is reversible. It is possible that exogenous NAD precursors push the equilibrium of this step in the opposite direction, increasing endogenous NMN production from NAD$^+$, though how intracellular NMN could be dephosphorylated to an NaR/NR reservoir in mammals is unknown. This concept of increased NAD breakdown during treatment with exogenous NMN in young animals is also supported by the increased formation of unlabelled Nam in plasma (Fig. 5o, 6o). Further, while labelled NMN treatment in vitro (Fig. 3) results in the formation of intact labelled NAD$^+$, this occurs at the cost of unlabelled NAD$^+$, for a net zero change in total NAD levels (Fig. 3c), suggesting that when NAD$^+$ is replete, the utilisation of exogenous NMN into newly synthesised NAD$^+$ results in a commensurate breakdown of existing material – potentially explaining the formation of unlabelled metabolites such as NR during treatment with exogenous, labelled NMN.
Rather than degrading or recycling existing metabolites into NR, another possibility is that exogenous NMN or NR could trigger a currently unknown step in mammals that leads to endogenous NR production. While NR can be produced by the reversible phosphorolysis of Nam and ribose-5-phosphate by a purine nucleophosphorylase (PNP) in *E. coli*\(^{61}\), this step is irreversible in mammals\(^{56,62,63}\), and other potential steps involved in endogenous NR production in mammals are unknown. Further work is needed to understand how this occurs, for example, whether it is due to the acute up-regulation of NAD\(^+\) biosynthetic enzymes, the time-scale by which this increase occurs, and a direct comparison of different isotope labelled NAD\(^+\) precursors to identify which metabolites trigger the production of endogenous metabolites under normal circumstances and during depletion of the microbiome. Regardless, the ability to trigger the production of endogenous NAD metabolites could explain why exogenous NR and NMN treatment lead to differences in pharmacokinetics, metabolite production and therapeutic effects when compared to Nam alone\(^ {48-50}\), despite their rapid metabolism into free Nam by the liver\(^ {45}\) (Fig. 4, 5).

Another speculative idea is the existence of a signalling pathway in the GIT that is sensitive to both exogenous NAD precursors and to microbial metabolites, which can mediate endogenous NAD metabolism. Metabolite sensing members of the G-protein coupled receptor (GPCR) family are putative candidates for this role as there are already GPCRs known to respond to extracellular nicotinic acid and to NAD\(^+\) itself\(^ {64}\). One possible candidate is GPR109a, which acts as a receptor both for nicotinic acid\(^ {65}\) and for butyrate, released from the microbial fermentation of dietary fibre\(^ {66}\). This could link the observations including the deamidation of orally derived NAD precursors into their acid equivalents, a role for microbiome depletion in triggering the production of endogenous NAD metabolites, and evidence for the poor incorporation of intact NR or NMN into the NAD metabolome. Identifying cell surface receptors and downstream signalling pathways in the gut that are sensitive to both microbial metabolites and exogenous NAD\(^+\) precursors will be a key goal of testing our hypothesis around the role of exogenous metabolites in endogenous NAD\(^+\) metabolism. Together,
these findings regarding the deamidation of NMN and its effects on the endogenous NAD metabolome have profound importance for the therapeutic development of NAD precursors.
Methods
Methods are available in Supplementary Material, with raw data available on our Mendeley data site.

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Author contributions
LJK conducted experiments, analysed data, prepared figures, wrote manuscript. TJC conducted microbiome analyses. EWKP, TTC, JW prepared isotope labelled NMN. SPT and DAS provided critical feedback and interpretation. LEQ conducted experiments, extracted and analysed data, wrote manuscript. LEW conceived of and designed study, obtained funding, supervised experiments, analysed data, prepared figures, wrote manuscript.

Declaration of interests
EWKP and JW are employees and shareholders of GeneHarbor Biotechnologies. SPT is the CEO of Jumpstart Fertility, which is developing NAD\(^+\) raising compounds for therapeutic use. LEW and DAS are co-founders, shareholders, directors and advisors of Jumpstart Fertility and the Life Biosciences group which includes Jumpstart Fertility, Continuum Biosciences, Senolytic Therapeutics, Selphagy, and Animal Biosciences. LEW and DAS are also advisors to and shareholders in the EdenRoc group of companies, which includes Metro Biotech NSW and Metro International Biotech, Arc-Bio, Dovetail Genomics, Claret, Revere Biosciences, and Liberty Biosecurity. LEW is an advisor and shareholder in Intravital Pty Ltd. DAS is an inventor on a patent application that has been licensed to Elysium Health. Updated affiliation are at https://genetics.med.harvard.edu/sinclair-test/people/sinclair-other.php.
Figure 1. NMN treatment leads to the microbiome-dependent formation of deamidated NAD$^+$ metabolites *in vivo*. Mice treated with antibiotics (Abx) to ablate the gut microbiome were administered a single dose of unlabelled nicotinamide mononucleotide (NMN) (500 mg/kg, oral gavage). Gastrointestinal tissue (GIT) (a-c) and liver tissue (d-f) were subject to targeted mass spectrometry to quantify the deamidated metabolites (a-f) nicotinic acid riboside (NaR) (a, d), nicotinic acid mononucleotide (NaMN) (b, e) and nicotinic acid adenine dinucleotide (NAAD) (c, f), as well as their amidated counterparts (g-l) nicotinamide riboside (NR) (g, j), NMN (h, k) and nicotinamide adenine dinucleotide (NAD$^+$) (i, l). These data were then expressed as ratios between
de-amidated and amidated counterparts in GIT (m-o) and liver (p-r). Data analysed by 2-way ANOVA with Sidak’s post-hoc test, exact p-values and F values in supplementary files. N=4-5 animals per group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 2. Scheme for assimilation of isotope labelled NMN into the NAD$^+$ metabolome.

NMN (box on left) was labelled with $^{13}$C at all 5 carbon positions of the ribose moiety (highlighted in red) and with $^{15}$N at the base (highlighted in blue) and amine (highlighted in orange) of the nicotinamide moiety for a total mass shift of M+7. The expected mass shift for each species is shown, with steps catalyzed by mammalian enzymes in solid black lines, and steps that are only known to be carried out by bacterial enzymes shown with dashed lines.
Figure 3. $^{15}$N-glutamine labelling of NAD$^+$ synthesis. Primary hepatocytes were treated with NMN1 (M+6) or NMN2 (M+7) isotopes (200 µM) in the presence of unlabelled or amide labelled $^{15}$N-glutamine (M+1) (4 mM) for 24 hr, to measure the degree of NAD synthesis and the incorporation of exogenous NMN via the de novo pathway, which incorporates the amide label of $^{15}$N-Gln through the enzyme NAD synthetase (NADS). Exogenous NMN1 and NMN2 isotopes in
the presence of $^{15}$N-Gln led to the expected isotopic labelling of a) NR, b) NMN, c) NAD$^+$ d) Nam and e) Gln. Ratios of f) M+1$_{\text{amide}}$ labelled to unlabelled NAD, g) M+7 labelled NAD$^+$, h) M+2 labelled NAD$^+$ and i) M+1$_{\text{amide}}$ Nam were consistent with labelling by $^{15}$N-Gln, with no change in j) M+1$_{\text{base}}$ labelled Nam acting as a negative control, as no change in labelling at the ring position is expected. This resulted in l) M+2 labelled Nam during NMN1 and 15N-Gln treatment. Data analysed by 2-way ANOVA with Sidak’s post-hoc test, n=3 biological replicates.
Figure 4. NMN deamidation in bacteria. Liquid cultures of *E. coli* OP50 bacteria were supplemented with M+6 labelled NMN1 (0.1 mM) at inoculation of a fresh culture. Samples were taken at time 0 (after NMN), 140, 160 and 180 minutes after NMN supplementation. Following separation of the culture supernatant (top) from the cell lysate (bottom), metabolites were extracted and subjected to targeted LC-MS/MS mass spectrometry to detect the incorporation of the M+6
isotope label into NMN, NR, NaMN and NAD$^+$ as well as M+1 labelling of nicotinamide (Nam) and nicotinic acid (Na) in both the culture supernatant (top) and cell lysates (bottom). Data represents mean ± s.d. (n=3-5 samples per time point).
Figure 5. Incorporation of NMN1 (M+6) isotope into the NAD metabolome in vivo. Animals were treated with antibiotics (Abx) to deplete the microbiome, followed by an oral gavage (50 mg/kg) of NMN1 (M+6) with adjacent i.p. administration of $^{15}$N-Gln (735 mg/kg, 10ml/kg body weight). Four hr later, GIT (a-g), liver (h-n) and plasma (o) were rapidly preserved for targeted metabolomics analysis to identify labelling of (a, h, o) Nam, (b, i) NMN, (c, j) NR, (d, k) NAD$^+$, (e, l) NaMN, (f, m) NaR and (g, n) NaAD. Data presented as stacked bars of each isotopologue, with raw data points for each isotopologue overlaid on bar charts. (p, q) Relative molar abundance of all NAD metabolites in (p) GIT and (q) liver, including endogenous (M+0), intact labelled and partially labelled (“part”) isotopologues. n=3-4 animals per group, n.d. = not detected.
Figure 6. Incorporation of NMN2 (M+7) into the NAD metabolome in vivo. Animals were treated with antibiotics (Abx) to deplete the microbiome, followed by an oral gavage (50 mg/kg) of NMN2 (M+7). Four hr later, GIT (a-g), liver (h-n) and plasma (o) were rapidly preserved for targeted metabolomics analysis to identify labelling of (a, h, o) Nam, (b, i) NMN, (c, j) NR, (d, k) NAD+, (e, l) NaMN, (f, m) NaR and (g, n) NaAD. Data presented as stacked bars of each isotopologue, with raw data points for each isotopologue overlaid on bar charts. (p, q) Relative molar abundance of all NAD metabolites in (p) GIT and (q) liver, including endogenous (M+0), intact labelled and partially labelled (“part”) isotopologues. n=3-4 animals per group n.d. = not detected.
Figure 7. Contribution of the microbiome to NMN deamidation. Antibiotic treated animals were orally administered with the a) NMN1 (M+6) isotope in the presence or absence of $^{15}$N-glutamine (M+1), with the formation of M+7 labelled NAD+ or M+2 labelled Nam reflective of incorporation following deamidation and reamidation by the enzyme NADS. Antibiotic treatment reduced (b, c)
M+7 labelling of NAD$^+$, (d-f) M+2 labelling of nicotinamide (Nam), (g, h) M+2 labelling of recycled NMN, and (i-j) M+2 labelling of recycled NAD$^+$, which reflects the incorporation of recycled Nam. Data are expressed as ratios to M+6 or M+1 NAD$^+$ and M+1$_{\text{base}}$ Nam, which are the expected isotope products of NMN or NR assimilation via the canonical (amidated) route. Incorporation of this extra label was limited by the availability of $^{15}$N-glutamine (M+1) as k) a proportion of the endogenous glutamine pool in plasma. In a separate cohort, animals were treated with l) NMN2 (M+7), where loss of the amide $^{15}$N label to form M+6 labelled NAD$^+$ or M+1$_{\text{base}}$ labelled Nam would reflect deamidation. Antibiotic treatment protected M+7 NAD$^+$ (m, n), M+2 Nam (o-q), and M+2 NAD$^+$ (n, o) against loss of the NMN2 $^{15}$N amide compared to untreated animals, which carried into M+2 labelling of recycled NMN, and (i-j) M+2 labelling of recycled NAD$^+$. Tissues shown in the left column (b, d, g, l, m, o, r, t) are gastrointestinal tract (GIT), middle column (c, e, h, j, n, p, s, u) are liver, right column (f, k, q) are plasma. NMN1 and NMN2 experiments were run in separate cohorts of animals, each measurement represents tissue from a separate animal. Comparisons of isotope treated groups with or without antibiotic treatment were analysed by Mann-Whitney U-test, *p=0.0286, n=4 animals per group.
Figure 8

Isotope labelled NMN treatment results in greater labelling of the NR than NMN pool, suggesting indirect uptake. (a) The two proposed mechanisms for NMN uptake are either directly through the putative NMN transporter SLC12A8, or indirectly by dephosphorylation into NR via the ecto-5’-nucleotidase CD73 which is present on the apical side of intestinal cells. To compare the contributions of either direct or indirect transport mechanisms, the contribution of isotope labelled NMN to the overall pool of each metabolite is shown for the intestinal tissue of the NMN2 cohort (data from Fig. 6). Error bars are s.d., each data point represents tissue from a separate animal.
Figure 9. Effects of chronic NMN treatment on the aged gut microbiome. Aged (97-week old) male and female mice were treated with NMN through addition to drinking water (~400 mg/kg/day) for 8-10 weeks prior to cull, and faecal microbiome samples subject to long-read 16S rRNA sequencing. (a) Stacked bar plots represent the total reads and relative abundance of bacterial taxa at the genus level for untreated or NMN treated animals, also shown as differential expression by the (b) genus level and (c) by operational taxonomic unit (OTU) level. There was no change in microbiome diversity, shown by the (d) Shannon alpha diversity index.
SUPPLEMENTARY FIGURES
Supplementary Figure 1. Antibiotics treatment ablates the gut microbiome. Following antibiotic treatment in the NMN1 and NMN2 mouse cohorts, (a-b) DNA was extracted from faeces to measure changes in DNA concentration. Uniform amounts of DNA were then subject to (c-d) full-length 16S rRNA Nanopore sequencing, with species abundance shown here at the genus level. Sequencing revealed a reduction in (e-f) operational taxonomic units (OTUs), the (g-h) Chao1 and (i-j) ACE species richness indices, and the (k-l) Shannon, (m-n) Simpson, (o-p) Inverse Simpson and (q-r) Fisher diversity indices. Data shown are non-rarefied; rarefication showed identical results (data not shown). Each data point represents samples from a separate animal.
Supplementary Figure 2. Chromatogram of isotope labelled NMN and glutamine (Gln) using MRM LC-MS/MS. The above chromatograms represent individual peaks (ion count) for a) 100µM unlabelled NMN in combined NAD metabolite standard curve mixture, b) M+6 labelled NMN1 and c) M+7 labelled NMN2, as well as d) unlabelled and 15N-amide labelled glutamine.
Supplementary Figure 3. Incorporation of NMN1 (M+6) into the GIT (raw data for Fig. 5). As in Figure 4 of main text, animals were treated with antibiotics (Abx) and M+6 isotope labelled NMN1 followed by metabolomics analysis of intestinal tissue as described in Figure 1 of main text. Animals
were also intraperitoneally injected with a concurrent bolus of $^{15}$N-amide labelled glutamine (735 mg/kg, 10ml/kg body weight). The left column (a-g) represents the abundance of unlabelled, endogenous species, middle column (h-n) represents intact label incorporation, right column (o-u) represents partial labelled species from the recycling of metabolites, as predicted in Fig. 2a. Data analysed by 2-way ANOVA with Sidak’s post-hoc test. n=3-4 animals per group, each data point represents a separate animal, error bars are SD.
Supplementary Figure 4

As in Figure 4 of main text, animals were treated with antibiotics (Abx) and M+6 isotope labelled NMN1 followed by metabolomics analysis of intestinal tissue as described in Figure 1 of main text.
Animals were also intraperitoneally injected with a concurrent bolus of $^{15}$N-amide labelled glutamine (735 mg/kg, 10ml/kg body weight). The left column (a-g) represents the abundance of unlabelled, endogenous species, middle column (h-n) represents intact label incorporation, right column (o-u) represents partial labelled species from the recycling of metabolites, as predicted in Fig. 2a. Data analysed by 2-way ANOVA with Sidak’s post-hoc test. n=3-4 animals per group, each data point represents a separate animal, error bars are SD.
Supplementary Figure 5. Incorporation of NMN2 (M+7) into the GIT (raw data for Fig. 6).

Animals were treated with antibiotics (Abx) and M+7 isotope labelled NMN2 followed by metabolomics analysis of GIT tissue as described in Figure 6 of main text. Each data point represents...
measurements from a different animal. The left column (a-d) represents the abundance of unlabelled, endogenous species, middle column (e-h) represents intact label incorporation, right column (i-l) represents partial labelled species from the recycling of metabolites, as predicted in Fig. 1. Metabolites assayed are nicotinamide riboside (NR), nicotinamide adenine dinucleotide (NAD$^+$), nicotinamide (Nam) and nicotinamide mononucleotide (NMN). Data are analysed by two-way ANOVA with Sidak’s multiple comparisons test. Data are mean ± s.d. (n=3-5 mice per group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.
Supplementary Figure 6

Animals were treated with antibiotics (Abx) and M+7 isotope labelled NMN2 followed by metabolomics analysis of liver tissue as described in Figure 6 of main text. Each data point represents
measurements from a different animal. The left column (a-d) represents the abundance of unlabelled, endogenous species, middle column (e-h) represents intact label incorporation, right column (i-l) represents partial labelled species from the recycling of metabolites, as predicted in Fig. 1. Metabolites assayed are nicotinamide riboside (NR), nicotinamide adenine dinucleotide (NAD$^+$), nicotinamide (Nam) and nicotinamide mononucleotide (NMN). Data are analysed by two-way ANOVA with Sidak’s multiple comparisons test. Data are mean ± s.d. (n=3-5 mice per group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.
SUPPLEMENTARY METHODS
Synthesis of isotope labelled NMN

The isotopes used here were generated through a two-step process starting with the custom synthesis of nicotinamide labelled with $^{15}$N at the nitrogen base and amide positions. This custom isotope labelled version of nicotinamide was then used with [U5]-$^{13}$C- ribose which was $^{13}$C labelled at all five carbon positions (Cambridge Isotope Laboratories, cat. no. CLM-3652) and ATP in an enzyme-based protocol using recombinant phosphoribosyl synthetase (PRS) and recombinant nicotinamide phosphoribosyl transferase (NAMPT) to synthesise NMN. The two enzymes were added into the reaction buffer that contains 1 mM ribose, 1 mM nicotinamide, 3 mM ATP, 1 mM dithiothreitol, 10 mM MgCl$_2$ and 50 mM Tris-HCl (pH 7.5) and incubated at 37°C for 30 min. The reaction was terminated with the addition of 0.01% Trichloroacetic acid (TCA). The purification was proceeded with size-exclusion columns and ion exchange columns. Isotope labelled NMN samples of >95% purity were concentrated by lyophilization, and labelling confirmed by mass spectrometry (Supp. Fig. 2).

Animal experiments

All experiments were performed according to procedures approved by UNSW Animal Care and Ethics Committee (ACEC) under ethics protocol 18/134A. The UNSW ACEC operates under the animal ethics guidelines from the National health and Medical Research Council (NHMRC) of Australia. Mice were fed standard chow ad libitum and housed under a 12-hr light/12-hr dark cycle in a temperature-controlled room (22 ± 1°C) at 80% humidity in individually ventilated cages. Four-week old female C57BL/6J mice were acclimatised for one week prior to treatment and body weight matched before random assignment into groups. For antibiotic treatment, mice were administered a cocktail of antibiotics consisting of vancomycin (0.5 g/L; Sigma SBR00001), neomycin (1 g/L; Sigma N6386), ampicillin (1 g/L; Sigma A9393) and metronidazole (1 g/L; Sigma, M3761) (VNAM) with addition of sucrose (3 g/L; Bundaberg Sugar) to increase palatability for 4 days, and switched to ampicillin (1 g/L) with sucrose (3 g/L) for an additional week, which can reduce gut bacterial...
density by 1000-fold\textsuperscript{67}. During treatment with the VNAM combination there was a reduction in water consumption (below), which was the reason for the subsequent switch to ampicillin alone. Sucrose treatment (3 g/L) was maintained as a vehicle control in animals that did not receive antibiotic treatment.

\begin{center}
\includegraphics[width=\textwidth]{water_intake_body_weight}
\end{center}

\textit{Above: Water intake and body weight of mice during antibiotics treatment.}

To maintain consistency between the three cohorts presented here (unlabelled NMN – Fig. 1, NMN1 – Fig. 5, NMN2 – Fig. 6), this antibiotic treatment protocol was used for all \textit{in vivo} experiments (Figs. 1, 5, 6). For NMN treatment, mice received a single oral gavage of NMN1 (Fig. 5) or NMN2 (Fig. 6) isotopes at 50 mg/kg, or for unlabelled NMN (Fig. 1) at 500 mg/kg, with water vehicle used as a control. Four hours later, animals were placed under anaesthesia, and blood was obtained by cardiac puncture, followed by euthanasia by cervical dislocation, rapid dissection and snap freezing of tissues. Gavages were staggered between mice in alternating treatment groups to avoid any experimental bias. On the day of cull mice were all 5-6 weeks old. Differences in NMN dosing between unlabelled and isotope labelled NMN were due to limited availability of isotope labelled NMN.

**Blood plasma collection and preparation for mass spectrometry**

Approximately 1 mL of blood was collected via cardiac puncture in anaesthetised mice (1-2% isoflurane) into 1.5 mL eppendorf tubes prefilled with 10 µL of EDTA (0.5 M) and mixed thoroughly
with a pipette to prevent clotting. Blood samples were then spun at 2000 g for 10 min and the top layer was transferred to a new tube and snap frozen immediately in liquid nitrogen. All samples were stored in -80°C until further processing. On the day of sample acquisition plasma samples were thawed on ice and 20 µL of plasma was added to 80 µL of extraction buffer (acetonitrile:methanol) with an internal standard mixture containing MES, CSA and thymine-d4. Samples were vortexed and centrifuged at 16,000 g for 10 mins at 4°C and the supernatant was transferred to a new eppendorf tube and dried down completely using a speed vacuum concentrator (Savant SpeedVac ® SPD140DDA, Thermo Scientific). The resulting pellet was then resuspended in 30 µL of LC-MS-grade water and centrifuged as above and the supernatant analysed promptly by LC-MS.

**Gastrointestinal and liver tissue collection and preparation for mass spectrometry**

Intestinal contents (small intestine and colon without cecum) were resected and flushed with ice cold 1 x phosphate buffered saline (PBS) to clear faecal contents before snap freezing immediately with liquid nitrogen. Livers were resected and weighed before being rinsed in ice cold 1x PBS and snap frozen in liquid nitrogen. All tissue samples were stored in -80 °C until further processing. Frozen tissue samples were crushed using a mortar and pestle on liquid nitrogen and approximately 50 mg was weighed into tubes containing ceramic beads (Precellys, Bertin Technologies, France) to which 500 µl of cold (-30 °C) extraction buffer (acetonitrile:methanol:water, 2:2:1) with internal standard mixture as above was added. All samples were homogenised using an automated tissue homogeniser (Precellys24, Bertin Technologies, France) at 5,000-6,000 rpm for 15 seconds and immediately centrifuged at 16,000g for 10 mins at 4 °C. The supernatant was transferred to a new tube and dried down completely using a speed vacuum concentrator (Savant SpeedVac ® SPD140DDA, Thermo Scientific). All samples were resuspended in 50 µL of LC-MS-grade water, centrifuged as above and the supernatant analysed promptly by LC-MS.
**Bacterial culture and NMN treatment**

A stab culture of the *E. coli* strain OP50 was inoculated into sterile Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast and 10 g/L sodium chloride in deionized water) under aseptic conditions and incubated overnight at 37°C on a shaking platform set at 200 rpm. To measure the growth rate of *E. coli*, the overnight culture was sub-cultured (1:200) into sterile LB broth in a new flask and the optical density was measured at 600 nm (OD$_{600}$) every 20 minutes (approximate doubling time) and samples were collected during the early-mid exponential growth phase (OD600 < 0.70), as bacterial enzymes are more active during exponential growth phase than stationary phase. For samples, the overnight culture was sub-cultured (1:200) and aliquoted into smaller volumes. The cultures were then supplemented with either vehicle (water) or M+6 labelled NMN (0.1 mM) and OD$_{600}$ measured at time zero (before NMN), time zero (after NMN), and 140, 160 and 180 minutes after supplementation with NMN. The supernatant of cells was separated from the cells via centrifugation (5000 g for 10 minutes at 4°C) and stored immediately at -30°C. Meanwhile, the cell pellet was resuspended in cold (4°C) saline solution (0.9% NaCl) and centrifuged as above, to rinse away residual media before storage at -30°C. The OD$_{600}$ was measured for each sample and used to normalise metabolite levels after LC-MS/MS analysis.

**Primary hepatocyte culture**

Primary hepatocytes were obtained as described previously. Male Sprague Dawley rats (250 grams, Animal Resources Centre, Perth, WA, Australia) were maintained on a 12:12 h day-night cycle, with water and food supplied *ad libitum*. Under deep non-recoverable general anaesthesia (75 mg/kg ketamine, 10 mg/kg xylazine, intraperitoneal administration) rats underwent laparotomy. The portal vein was cannulated *in situ* and the liver perfused initially with carbogen-saturated perfusion media (final: NaCl 138 mM, HEPES 25 mM, D-glucose 5.6 mM, KCl 5.4 mM, Na$_2$HPO$_4$ 0.34 mM, KH$_2$PO$_4$ 0.44 mM, NaHCO$_3$ 4.17 mM, EDTA 0.5 mM, pH 7.4, 37°C, 25 ml/min flow rate). The inferior vena cava was cut to allow efflux. After 4 mins, the carbogen-saturated perfusion media was
changed to the collagenase containing buffer (final: NaCl 138 mM, HEPES 25 mM, D-glucose 5.6 mM, KCl 5.4 mM, Na₂HPO₄ 0.34 mM, KH₂PO₄ 0.44 mM, NaHCO₃ 4.17 mM, CaCl₂ 2 mM, collagenase II (Sigma, 15950-017), pH 7.4, 37°C, 25 ml/min flow rate) for 6 mins. The inferior vena cava was clamped at least 10 times during the collagenase digestion (preventing efflux) resulting in liver swelling that allows a better digestion.

Following the collagenase digestion, the liver was removed and place on ice in 20 ml Williams’ Medium E (Life-technologies, Waltham, MA, USA). The hepatocytes were gently dispersed in the medium and the cells filtered through a 100 µm cell strainer. Hepatocytes were washed and diluted Williams’ Medium E and plated (6-well plates) at 10⁶/2ml/well. After 4 hrs of incubation the culture medium was changed to MOPS buffer (final, NaCl 128 mM, MOPS 23.9 mM, KCl 6 mM, MgSO₄·7H₂O 1.18 mM, CaCl₂ 1.29 mM, glucose 5 mM, BSA (FFA) 0.2 %, pH 7.4) and cells incubated overnight. Following overnight incubation, cells were incubated in M199 media without glutamine (Sigma M2154), supplemented with either unlabelled (Sigma) or ¹⁵N-amide labelled glutamine (Cambridge Isotope Laboratories NLM-557) at 4 mM, in the presence or absence of NMN1 or NMN2 isotopes at 200 µM for 24 hr, following which samples were preserved for metabolomic analysis (Fig. 3).

**Preparation of NAD⁺ metabolite standards**

NAD⁺ metabolites were serially diluted starting from a concentration of 100 µM to 0.39 µM. The same volume (500 µL) of extraction buffer (acetonitrile:methanol:water) was added and vortexed before centrifuging and transferring to new tube ready to be dried down as above. The subsequent steps were the same as preparing the tissue samples as above. All standards and samples were processed on the same day to reduce any experimental bias or variability. Standard curves used to calculate absolute concentrations are shown on the following page (Methods Fig. 2) and are available in supplementary raw data files.
Above: NAD+ metabolite standard curves. (a) NMN (b) Nam (c) NAD+ (d) NR (e) Na (f) NaMN (g) NaAD (h) NaR. Standard curves were serially diluted from 50 µM to 0.39 µM. Shown here from 50 µM to 0.39 µM for all metabolites except for NR which is shown from 50 mM to 0.39 µM, due to the unexpectedly high concentrations of NR in the GIT (Fig. 1, 5, 6) and NaR from 100 µM to 0.39 µM.

**Mass spectrometry**

The LC-MS method was performed using 1260 Infinity LC System (Agilent) coupled to QTRAP 5500 (AB Sciex) mass spectrometer. LC separation by gradient elution was accomplished on an XBridge BEH amide column (100 mm x 2.1 mm, 3.5 µm particle size, Waters Corporation) at room
temperature. For the mobile phase, Solvent A is 95%:5% H$_2$O:acetonitrile containing 20 mM ammonium acetate and 20 mM acetic acid, and solvent B is acetonitrile. The flow rate was 200 µL/min, with the percentage of solvent B set at 85% (0 min), 85% (0.1 min), 70% (10 min), 15% (13 min), 15% (17 min), 85% (17.5 min), 85% (30 min) (Supplementary Table 1). Injection volume was 2.5 µL. Ion source was set at 350 °C and 4500 V with polarity switching. Mass isotopologues of metabolites were acquired by MS$^2$, using the unscheduled multiple reaction monitoring (MRM) mode with a dwell time of 40 ms. The MS parameters (declustering potential, collision energy and cell exit potential) (Supplementary Table 2a) and MRM transitions were calibrated based on the monoisotopic mass of chemical standards (Supplementary Figure 11). Data processing was performed using MSConvert (version 3.0.18165-fd93202f5) and in-house MATLAB scripts. Deconvolution scripts were developed to resolve overlapping NAAD-NAD, NAR-NR and NAMN-NMN peaks using MATLAB’s Optimisation Toolbox. Representative chromatograms are shown below.

Left: chromatograms represent individual peaks (ion count) from 100µM standard solutions at each respective retention times for **(a)** NAM, NA, MeNAM, NAD$^+$ and NAAD, **(b)** NAMN and NMN, **(c)** NAR and NR.
Statistical analysis for mass spectrometry

All data are presented as mean ± standard deviation (s.d.). Statistical significance was performed using a two-way ANOVA with a Sidak’s multiple comparisons test to determine differences between groups after removing outliers using the ROUT method (Q=1%). Data in Fig. 7 were analysed by Mann-Whitney U-test between NMN isotope treated groups due to the absence of detection of labelled metabolites in animals that did not receive NMN isotopes. All statistics were performed on GraphPad Prism software (version 8.2.1). P values less than 0.05 were considered statistically significant. All data analyses are available as an .xml file available on our Mendeley data site. For in vivo experiments (Fig. 1, 5-8, Supp. Fig. 4-7), each data point represents tissues from a separate animal, while each data point for in vitro experiments (Fig. 2, 3) represents an independent biological replicate.

DNA extraction from faecal pellets

Solid faecal pellets taken from the colonic and rectal region of the gastrointestinal tract were stored in -80°C until further processing. DNA was extracted from frozen faecal pellets using the QIAamp® PowerFecal® DNA kit (Qiagen, Cat. No. 12830-50) according to the manufacturer’s protocol. DNA concentration was determined using the NanoDrop™ (DeNovix®, DS-11 FX) and the purity of double-stranded DNA (dsDNA) was also determined by measuring the 260/280 ratio. All DNA extracts were stored at -80°C until further processing by 16S rRNA sequencing.

16S Sequencing

Full length 16S rRNA genes were amplified by PCR using the Oxford Nanopore 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK). Briefly, 10 ng genomic DNA, 1 µL 16S Barcode (10 µM) and 25 µL LongAmp Taq 2X Master Mix (New England Biolabs, Ipswich, MA, USA) were combined in a 50 µL reaction for PCR on a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). PCR cycling condition were as follows; initial
denaturation at 95 °C for 1 minute, 25 cycles of denaturation at 95 °C for 20 seconds, annealing at 55 °C for 30 seconds and extension at 65 °C for 2 minutes before a final extension at 65 °C for 5 minutes. PCR products were purified as per Oxford Nanopore Technologies (ONT) protocol using AMPure XP magnetic beads (Beckman Coulter, Indianapolis, IN) and DNA quantified using the NanoDrop™ (DeNovix®, DS-11 FX). Barcodes were pooled to a total of 100 fmol in 10 μL of 10 mM Tris-HCl, pH 8.0 with 50 mM NaCl for library loading. Sequencing was performed using R9.4.1 ONT Flow Cells on the MinION™ sequencing platform and data acquired using MinKNOW software version 19.10.1 (Oxford Nanopore Technologies).

**Data Analysis for 16S sequencing**

Full length 16S sequencing reads acquired from MinION runs (i.e. FAST5 data) were base-called to fastq files using Guppy software version 3.4.4 (Oxford Nanopore Technologies). Fastq files were demultiplexed using Porechop (https://github.com/rrwick/Porechop) and trimmed to 1400bp with Trimmomatic version 0.39. Reads were imported to QIIME2 for dereplication and chimeric reads screened and filtered from the dataset. Operational taxonomic unit clustering was completed within QIIME2 version 2019.7.0 at 85% similarity to account for typical sequencing errors obtained from long-read sequencing. Taxonomy was assigned to reads using a pre-trained classifier on the SILVA 132 16S rRNA representative sequences. Data was imported into R version 3.6.1 with qiime2R version 0.99.13 (https://github.com/ibisanz/qiime2R) for visualisation and alpha diversity analysis using raw and rarefied data with the phyloseq version 1.30.0 package. Scripts for command line processing and analysis in R available in Supplementary Materials. Sequencing data has been deposited in the NCBI database Sequence Read Archive (SRA) under accession numbers PRJNA635359.

**Figures**

Labelling schemes shown in Fig. 1-4, 7 were created using BioRender.com
SUPPLEMENTARY TABLES

Supplementary Table 1.

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Supplementary Table 2. Liquid chromatography (LC) separation gradient

**Buffer A:** 95:5 (v/v) HPLC H₂O:Acetonitrile (CH₃CN) with 20 mM ammonium acetate (NH₄OAc) + 20mM acetic acid (CH₃COOH), pH 5. **Buffer B:** 100% Acetonitrile (CH₃CN).
**Supplementary Table 2.**

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**Supplementary Table 2.** MRM transitions and MS parameters of NAD\(^+\) metabolites and MRM internal standards (Thymidine d4, CSA, MES). Q1: parent ion, Q3: fragment ion, MRM: multiple reaction monitoring, DP: declustering potential, CE: collision energy, CXP: collision cell exit potential.
References


NMN treatment leads to the microbiome-dependent formation of deamidated NAD+ metabolites in vivo. Mice treated with antibiotics (Abx) to ablate the gut microbiome were administered a single dose of unlabelled nicotinamide mononucleotide (NMN) (500 mg/kg, oral gavage). Gastrointestinal tissue (GIT) (a-c) and liver tissue (d-f) were subject to targeted mass spectrometry to quantify the deamidated
metabolites (a-f) nicotinic acid riboside (NaR) (a, d), nicotinic acid mononucleotide (NaMN) (b, e) and nicotinic acid adenine dinucleotide (NAAD) (c, f), as well as their amidated counterparts (g-l) nicotinamide riboside (NR) (g, j), NMN (h, k) and nicotinamide adenine dinucleotide (NAD+) (i, l). These data were then expressed as ratios between de-amidated and amidated counterparts in GIT (m-o) and liver (p-r). Data analysed by 2-way ANOVA with Sidak’s post-hoc test, exact p-values and F values in supplementary files. N=4-5 animals per group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Scheme for assimilation of isotope labelled NMN into the NAD+ metabolome. NMN (box on left) was labelled with 13C at all 5 carbon positions of the ribose moiety (highlighted in red) and with 15N at the base (highlighted in blue) and amine (highlighted in orange) of the nicotinamide moiety for a total mass shift of M+7. The expected mass shift for each species is shown, with steps catalyzed by mammalian enzymes in solid black lines, and steps that are only known to be carried out by bacterial enzymes shown with dashed lines.
15N-glutamine labelling of NAD+ synthesis. Primary hepatocytes were treated with NMN1 (M+6) or NMN2 (M+7) isotopes (200 µM) in the presence of unlabelled or amide labelled 15N-glutamine (M+1) (4 mM) for 24 hr, to measure the degree of NAD synthesis and the incorporation of exogenous NMN via the de novo pathway, which incorporates the amide label of 15N-Gln through the enzyme NAD synthetase (NADS). Exogenous NMN1 and NMN2 isotopes in the presence of 15N-Gln led to the expected isotopic labelling of a) NR, b) NMN, c) NAD+ d) Nam and e) Gln. Ratios of f) M+1amide labelled to unlabelled NAD, g) M+7 labelled NAD+, h) M+2 labelled NAD+ and i) M+1amide Nam were consistent with labelling by 15N-Gln, with no change in j) M+1base labelled Nam acting as a negative control, as no change in labelling at the ring position is expected. This resulted in l) M+2 labelled Nam during NMN1 and 15N-Gln treatment. Data analysed by 2-way ANOVA with Sidak's post-hoc test, n=3 biological replicates.
NMN deamidation in bacteria. Liquid cultures of E. coli OP50 bacteria were supplemented with M+6 labelled NMN1 (0.1 mM) at inoculation of a fresh culture. Samples were taken at time 0 (after NMN), 140, 160 and 180 minutes after NMN supplementation. Following separation of the culture supernatant (top) from the cell lysate (bottom), metabolites were extracted and subjected to targeted LC-MS/MS mass spectrometry to detect the incorporation of the M+6 isotope label into NMN, NR, NaMN and NAD+ as well
as M+1 labelling of nicotinamide (Nam) and nicotinic acid (Na) in both the culture supernatant (top) and cell lysates (bottom). Data represents mean ± s.d. (n=3-5 samples per time point).

Figure 5

Incorporation of NMN1 (M+6) isotope into the NAD metabolome in vivo. Animals were treated with antibiotics (Abx) to deplete the microbiome, followed by an oral gavage (50 mg/kg) of NMN1 (M+6) with adjacent i.p. administration of 15N-Gln (735 mg/kg, 10ml/kg body weight). Four hr later, GIT (a-g), liver (h-
n) and plasma (o) were rapidly preserved for targeted metabolomics analysis to identify labelling of (a, h, o) Nam, (b, i) NMN, (c, j) NR, (d, k) NAD+, (e, l) NaMN, (f, m) NaR and (g, n) NaAD. Data presented as stacked bars of each isotopologue, with raw data points for each isotopologue overlaid on bar charts. (p, q) Relative molar abundance of all NAD metabolites in (p) GIT and (q) liver, including endogenous (M+0), intact labelled and partially labelled (“part”) isotopologues. n=3-4 animals per group, n.d. = not detected.

Figure 6

Incorporation of NMN2 (M+7) into the NAD metabolome in vivo. Animals were treated with antibiotics (Abx) to deplete the microbiome, followed by an oral gavage (50 mg/kg) of NMN2 (M+7). Four hr later, GIT (a-g), liver (h-n) and plasma (o) were rapidly preserved for targeted metabolomics analysis to identify
labelling of (a, h, o) Nam, (b, i) NMN, (c, j) NR, (d, k) NAD+, (e, l) NaMN, (f, m) NaR and (g, n) NaAD. Data presented as stacked bars of each isotopologue, with raw data points for each isotopologue overlaid on bar charts. (p, q) Relative molar abundance of all NAD metabolites in (p) GIT and (q) liver, including endogenous (M+0), intact labelled and partially labelled (“part”) isotopologues. n=3-4 animals per group n.d. = not detected.
Contribution of the microbiome to NMN deamidation. Antibiotic treated animals were orally administered with the a) NMN1 (M+6) isotope in the presence or absence of 15N-glutamine (M+1), with the formation of M+7 labelled NAD+ or M+2 labelled Nam reflective of incorporation following deamidation and reamidation by the enzyme NADS. Antibiotic treatment reduced (b, c) M+7 labelling of NAD+, (d-f) M+2 labelling of nicotinamide (Nam), (g, h) M+2 labelling of recycled NMN, and (i-j) M+2 labelling of recycled NAD+, which reflects the incorporation of recycled Nam. Data are expressed as ratios to M+6 or M+1 NAD+ and M+1base Nam, which are the expected isotope products of NMN or NR assimilation via the canonical (amidated) route. Incorporation of this extra label was limited by the availability of 15N-glutamine (M+1) as k) a proportion of the endogenous glutamine pool in plasma. In a separate cohort, animals were treated with l) NMN2 (M+7), where loss of the amide 15N label to form M+6 labelled NAD+ or M+1base labelled Nam would reflect deamidation. Antibiotic treatment protected M+7 NAD+ (m, n), M+2 Nam (o-q), and M+2 NAD+ (n, o) against loss of the NMN2 15N amide compared to untreated animals, which carried into M+2 labelling of recycled NMN, and (i-j) M+2 labelling of recycled NAD+. Tissues shown in the left column (b, d, g, l, m, o, r, t) are gastrointestinal tract (GIT), middle column (c, e, h, j, n, p, s, u) are liver, right column (f, k, q) are plasma. NMN1 and NMN2 experiments were run in separate cohorts of animals, each measurement represents tissue from a separate animal. Comparisons of isotope treated groups with or without antibiotic treatment were analysed by Mann-Whitney U-test, *p=0.0286, n=4 animals per group.
Isotope labelled NMN treatment results in greater labelling of the NR than NMN pool, suggesting indirect uptake. (a) The two proposed mechanisms for NMN uptake are either directly through the putative NMN transporter SLC12A8, or indirectly by dephosphorylation into NR via the ecto-5'-nucleotidase CD73 which is present on the apical side of intestinal cells. To compare the contributions of either direct or indirect transport mechanisms, the contribution of isotope labelled NMN to the overall pool of each metabolite is
Effects of chronic NMN treatment on the aged gut microbiome. Aged (97-week old) male and female mice were treated with NMN through addition to drinking water (~400 mg/kg/day) for 8-10 weeks prior to cull, and faecal microbiome samples subject to long-read 16S rRNA sequencing. (a) Stacked bar plots represent the total reads and relative abundance of bacterial taxa at the genus level for untreated or NMN treated animals, also shown as differential expression by the (b) genus level and (c) by operational taxonomic unit (OTU) level. There was no change in microbiome diversity, shown by the (d) Shannon alpha diversity index.
Supplementary Files

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- Kimetal16SdifferentialabundanceFig8.xlsx
- Kimetal.2020statisticalanalyses.xlsx