Restoring an adequate dietary fiber intake by inulin supplementation: a pilot study showing an impact on gut microbiota and sociability in alcohol use disorder patients

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ABSTRACT
Alcohol use disorder (AUD) is a chronic relapsing disease associated with malnutrition, metabolic disturbances, and gut microbiota alterations that are correlated with the severity of psychological symptoms. This study aims at supplementing AUD patients with prebiotic fiber during alcohol withdrawal, in order to modulate the gut microbiota composition and to evaluate its effect on gastrointestinal tolerance, metabolism, and patient’s behavior. A randomized, double-blind, placebo-controlled study included 50 AUD patients assigned to inulin versus maltodextrin daily supplementation for 17 days. Biological measurements (fecal microbial 16S rDNA sequencing, serum biology), dietary intake, validated psychological questionnaires, and gastrointestinal tolerance assessment were performed before and after the intervention. Inulin significantly decreased the richness and evenness and induced changes of 8 genera (q < 0.1) including \textit{Bifidobacterium} and \textit{Bacteroides}. Prebiotic had minor effects on gastrointestinal symptoms and nutritional intakes compared to placebo. All patients showed an improvement in depression, anxiety, and craving scores during alcohol withdrawal regardless of the intervention group. Interestingly, only patients treated with inulin significantly improved the sociability score and had an increased serum level of brain-derived neurotrophic factor. This pilot study shows that inulin is well tolerated and modulates the gut microbiota and the social behavior in AUD patients, without further improving other psychological and biological parameters as compared to placebo. Gut2Brain study, clinicaltrial.gov: NCT03803709, https://clinicaltrials.gov/ct2/show/NCT03803709

Introduction
Alcohol use disorder (AUD) is a major public health problem affecting 5–10\% of the population in developed countries. AUD is associated with metabolic disturbances, nutritional imbalance and has deleterious effects on mental health.\textsuperscript{1,2} AUD patients are prone to develop emotional and cognitive symptoms that contribute to the persistence of addictive behavior and to the risk of relapse.\textsuperscript{3} Chronic alcohol consumption induces alterations in neurotransmission and it has been shown that alcohol consumption and appetite regulation share common neurobiological mechanisms with hormones (leptin, ghrelin) and neuromodulators (dopaminergic, opioidergic system) being involved in both eating and addictive behaviors.\textsuperscript{3–6} However, chronic ethanol exposure impacts other systems that could interact with the brain and therefore also influence behavior. Indeed, chronic alcohol consumption is associated with alterations in the composition and function of the gut microbiota.\textsuperscript{7–9} These changes include increased abundance of Lachnospiraceae while there is a decrease of some specific bacteria like \textit{Bifidobacterium} and \textit{Faecalibacterium prausnitzii}.\textsuperscript{7,10,11} Several studies demonstrated that alterations of gut function could have an impact on cognition, mood and behavior.\textsuperscript{12–15} We have previously established a
link between gut dysbiosis, intestinal permeability and the severity of psychological symptoms, such as depression, anxiety, alcohol craving but also social impairments, suggesting the involvement of the gut–brain axis in the etiology of AUD.⁷,¹⁶

Diet is one of the main modulators of the gut microbiota composition and function.¹⁷ AUD patients have reduced carbohydrate, protein and fat intakes, and their dietary fiber (DF) intake is also well below the recommended value.¹⁸–²⁰ Among DF, inulin-type fructans are interesting as they go along with the definition of prebiotics: “substrates that are selectively used by host microorganisms conferring a health benefit” meaning that they promote the growth of some specific bacteria.²¹ Inulin-type fructans are natural components present in several fruits and vegetables including wheat, onion, banana, garlic, Jerusalem artichoke, chicory and leek.²² Inulin is fermented in the colon and has been shown to promote the growth of Bifidobacterium and Faecalibacterium prausnitzii. ²³,²⁴ The effects of inulin on gut health and metabolism have been widely studied in the context of obesity and metabolic disorders. For instance, fructan supplementation improves gut barrier function, decreases serum lipopolysaccharides (LPS) and inflammatory cytokines in preclinical and clinical studies.²³,²⁵,²⁶ We have also shown that DF deficiency in AUD patients is associated with gastrointestinal discomfort and psychological alterations.²⁸ Therefore, inulin supplementation could be an interesting approach to increase dietary fiber intake and to modulate the gut microbiota in order to improve psychological symptoms of AUD patients.

The objective of the study was to test in a randomized, placebo-controlled design the effect of enhanced dietary fiber intake, through inulin supplementation, on gut microbiota composition gastrointestinal tolerance, mood, alcohol craving and biological markers of satiety, lipid and glucose homeostasis.

**Results**

**Study population**

Among the 50 enrolled patients, 4 dropped out of the study in the placebo group and 3 in the inulin group (Figure 1). The population therefore consisted of 21 patients with complete data in the placebo group and 22 in the inulin group at T2. Compliance with the study treatment was 96% in placebo and 98% in inulin group. The sociodemographic and clinical comparisons of the two groups are presented in Table 1. Patients from both groups were similar except for the DSM-5 score \((p = .01)\) and the number of alcohol withdrawal cures \((p = .04)\). The inulin group had on average 1 more criteria in the DSM-5 classification and underwent less previous alcohol withdrawal cures \((2.6 \pm 2.4\) in placebo vs \(1.4 \pm 0.80\) in inulin group). The patients in both groups were characterized by severe AUD \((DSM-5 \geq 6\) criteria) and no difference was found in terms of alcohol consumption, duration of drinking habits and age of loss of control. A gender imbalance was observed between the groups although it did not reach significance \((24\% of women in placebo group vs 50\% in inulin group; \(p = .11)\). Eight patients relapsed during the second week of the program (at home) in placebo group vs 12 in inulin group \((32\% vs 48\% respectively \(p = .25)\). The subjects who relapsed had consumed alcohol on 3 out of 7 days \((2.8 \pm 2.1\) in placebo vs \(3.4 \pm 1.9\) in inulin group, \(p = .45)\). Patients who relapsed in the placebo group consumed 79 g/d of ethanol on average vs 76 g/d in the inulin group \((p = .96)\).

**Inulin supplementation is well tolerated by AUD patients**

It has been shown that fermentable DF intake, such as inulin, could lead to bloating and discomfort in some individuals.²⁷,²⁸ We therefore carefully monitored the gastrointestinal tolerance of patients throughout the intervention. Gastrointestinal pain was assessed from the first day of hospitalization and then every other day from the beginning of treatment (day 3 to day 18). The results are presented in Figure 2. There was no difference for abdominal pain, bloating, satisfaction of transit or the impact of the symptoms on daily life between placebo and inulin groups (Figure 2a-d). The frequency and the consistency of stools were comparable between both groups of treatment (Figure 2e-f). Because it has been shown that functional gastrointestinal disorders are more prevalent in women than in men, we also took into account the gender.²⁹ Gender adjustment did not affect the results (Supplementary Table S1).
**Figure 1.** Flow chart of the Gut2Brain study.

**Table 1.** Baseline characteristics of study participants.

| Sociodemographic characteristics | Placebo n = 21 | Inulin n = 22 | p  |
|----------------------------------|---------------|---------------|----|
| **Age (y)**                      | 48.0 ± 9.0    | 48.4 ± 9.8    | .90|
| Gender, n (%)                    |               |               | .11|
| Male                             | 16 (76.2)     | 11 (50.0)     |    |
| Female                           | 5 (23.8)      | 11 (50.0)     |    |
| Marital status, n (%)            |               |               | .56|
| Couple/ married                  | 9 (42.9)      | 7 (32.0)      |    |
| Single                           | 8 (38.1)      | 12 (52.0)     |    |
| Separated/divorced               | 4 (19.0)      | 3 (16.0)      |    |
| Educational level, n (%)         |               |               | .73|
| Primary                          | 2 (9.5)       | 2 (9.1)       |    |
| Secondary                        | 8 (38.1)      | 6 (27.3)      |    |
| Superior                         | 11 (52.4)     | 14 (63.6)     |    |
| **Clinical examination**         |               |               |    |
| Weight (kg)                      | 71.5 ± 10.4   | 73.4 ± 14.7   | .64|
| BMI (kg/m²)                      | 23.5 ± 3.5    | 24.4 ± 3.1    | .34|
| MMSE score                       | 28.8 ± 1.2    | 27.7 ± 2.9    | .33|
| Smoking, n (%)                   | 17 (81.0)     | 16 (72.7)     | .72|
| **Alcohol history**              |               |               |    |
| DSM-5 AUD score                  | 7.9 ± 2.0     | 9.3 ± 1.4     | .02|
| Age of loss of control (y)       | 31.6 ± 10.6   | 31.9 ± 12.0   | .93|
| Number of alcohol withdrawal cures | 2.6 ± 2.4  | 1.4 ± .80     | .04|
| Duration of drinking habit (y)   | 15.7 ± 10.2   | 16.5 ± 11.9   | .95|
| Alcohol consumption (g/d)        | 127.9 ± 59.6  | 152.7 ± 90.7  | .54|

Values are means ± standard deviation. N = 43.

p values were calculated using a T-test or Mann Whitney Wilcoxon's test and Ch² test or Fisher's test for categorical variables.

AUD, Alcohol use disorders; Alcohol Use Disorders Test; BMI, Body mass index; DSM-5, Diagnostic and Statistical Manual of Mental Disorders fifth edition; MMSE, Mini Mental State Examination.
Inulin supplementation induces changes in gut microbiota composition at phylum, family, and genus level in AUD patients

Fecal samples were collected in 24 patients at T1 and in 19 at T2 for the placebo group and 22 patients at T1 and 19 at T2 for the inulin group. MANOVA with 9999 permutations performed on 4 beta-diversity indices (Bray-Curtis, Jaccard, Unweighted UniFrac and Weighted UniFrac) returned a non-significant p-value (Figure 3a). However, the α-diversity indexes highlighted that inulin induced a decrease in richness and evenness. Indeed, inulin supplementation decreased significantly the number of observed species as well as Chao1 and Shannon indexes (Figure 3b). The total bacteria amount, measured by qPCR, was not impacted by inulin supplementation (Figure 3c). Phylum and family levels of bacteria revealed changes in the inulin group (Figure 3d). Indeed, in this group, we observed a significant increase in Actinobacteriota phylum (q < 0.05) and Bifidobacteriaceae family (q < 0.05). We also observed a significant decrease in the Bacteroidaceae family in the inulin group. At the genus level, prebiotic treatment largely increased Bifidobacterium and decreased Bacteroides, Dorea and Ruminococcus torques group (q < 0.05; Table 2). We also observed a trend toward an increase in Faecalibacterium relative abundance.
(4.8 ± 3.2% at T1 vs 6.3 ± 4.4% at T2; \( p = .055 \); data not shown) after inulin supplementation. Inulin supplementation induced a significant increase in the abundance of *Bifidobacterium adolescentis* and *Bifidobacterium longum* (Supplementary Figure S1).

In the placebo group, some changes occurred (none of them at the q value), with a decrease of *Acidaminococcus*, *Sutterella*, *Oscillibacter*, *Escherichia-Shigella*, *Flavonifractor* and *Bifidobacterium* and an increase in *Lachnospiraceae* ND3007 group, *Lachnospiraceae* NK4A136 group, *Gordonibacter*, *Monoglobus*, *Oscillospiraceae_UCG-002* and *Oscillospira-ceae_UCG-003* (\( p < .05 \) and \( q > 0.1 \); Table 2).

**Inulin treatment marginally modulates food and drink intakes upon alcohol withdrawal in AUD patients**

Globally, inulin supplementation had only a minor effect on food intake (Table S2). Inulin reduced the consumption of roots and tubers despite a lack of significance in the within group comparison (\( \beta = -33.2, p = .02; \beta = -34.4, p = .03 \) in model 1 and 2 respectively; Table S2). Patients in inulin group increased their fruit or vegetable juice consumption compared to placebo (\( \beta = 185.8, p = .03; \beta = 202.5, p = .03 \) in model 1 and 2 respectively; Table S2). The placebo group increased bread (\( p = .001 \)), biscuit and cakes (\( p = .02 \)) and cheese (\( p = .009 \)) consumption.
while the inulin group increased dairy products (excluded cheese) consumption ($p = .03$). Both groups significantly increased sweets and soda intake ($+170$ g/d, $p = .001$ for the placebo group and $+254$ g/d, $p = .04$ for the inulin group), the difference between groups was not significant (Table S2). The consumption of raw fruits and coffee also increased, independently of the treatment group, during the withdrawal period (fruits: $+48$ g/d, $p = .03$ for placebo group and $+42$ g/d, $p = .02$ for inulin group; Coffee: $+0.18$ L/d, $p = .006$ for placebo group and $+0.16$ L/d, $p = .008$ for inulin group; Table S2).

The Supplementary Table S3 presents the total energy and macronutrient intake of AUD patients. During the second week of the program, at home, 20 patients relapsed (8 in placebo group vs 12 in...
Table 2. Significant changes in relative abundance of gut bacteria at the genus level in AUD patient receiving inulin or placebo for 3 weeks*.

|                | Placebo | Inulin | p Placebo | q Placebo | p Inulin | q Inulin |
|----------------|---------|--------|-----------|-----------|----------|----------|
| **Changed in placebo** |         |        |           |           |          |          |
| Acidaminococcus | 1.059 ± 1.460 | 0.660 ± 1.031 | 0.039 | 0.183 | 0.418 | 0.617 |
| Lachnospiraceae ND3007 group | 0.162 ± 0.154 | 0.210 ± 0.228 | 0.016 | 0.148 | 0.670 | 0.785 |
| Lachnospiraceae NK4A136 group | 0.043 ± 0.112 | 0.125 ± 0.305 | 0.017 | 0.148 | 1.000 | 1.000 |
| **Monoglobus** | 0.158 ± 0.202 | 0.450 ± 0.815 | 0.005 | 0.148 | 0.977 | 1.000 |
| **Oscillibacter** | 0.344 ± 0.344 | 0.206 ± 0.177 | 0.049 | 0.218 | 0.077 | 0.268 |
| **Escherichia Shigella** | 1.038 ± 2.391 | 0.220 ± 0.498 | 0.011 | 0.148 | 0.134 | 0.321 |
| **Eubacterium straum group** | 0.351 ± 0.577 | 0.822 ± 1.130 | 0.026 | 0.148 | 0.754 | 0.850 |
| **Sutterella** | 1.998 ± 1.512 | 1.377 ± 1.169 | 0.009 | 0.148 | 0.055 | 0.241 |
| **Oscillospiraceae UCG-002** | 1.852 ± 2.348 | 2.316 ± 2.411 | 0.021 | 0.148 | 0.098 | 0.284 |
| **Oscillospiraceae UCG-003** | 0.069 ± 0.083 | 0.138 ± 0.155 | 0.041 | 0.203 | 0.060 | 0.241 |
| **Gordonibacter** | 0.056 ± 0.085 | 0.221 ± 0.460 | 0.025 | 0.148 | 0.295 | 0.495 |
| **Dorea** | 0.261 ± 0.228 | 0.212 ± 0.174 | 0.651 | 0.781 | <0.001 | 0.007 |
| **Bacteroides** | 20.39 ± 10.40 | 19.34 ± 9.77 | 0.073 | 0.278 | 0.002 | 0.028 |
| **Ruminococcus torques group** | 0.456 ± 0.612 | 0.279 ± 0.330 | 0.296 | 0.460 | 0.001 | 0.027 |
| **Lachnospiraceae unknown genus** | 0.693 ± 0.438 | 0.622 ± 0.348 | 0.275 | 0.460 | <0.001 | 0.007 |
| **Haemophilus** | 0.015 ± 0.039 | 0.034 ± 0.066 | 0.575 | 0.743 | 0.021 | 0.180 |
| **Butyrivibrio** | 0.371 ± 0.243 | 0.460 ± 0.277 | 0.418 | 0.616 | 0.029 | 0.194 |
| **Desulfovibrio** | 0.816 ± 1.915 | 0.811 ± 1.004 | 0.277 | 0.825 | 0.030 | 0.194 |
| **Dialister** | 0.821 ± 1.976 | 0.634 ± 1.026 | 0.488 | 0.686 | 0.017 | 0.159 |
| **Opposite Changes** |         |        |           |           |          |          |
| **Bifidobacterium** | 5.275 ± 5.232 | 3.556 ± 3.263 | 0.026 | 0.148 | <0.001 | 0.007 |
| **Oscillospiraceae unknown genus** | 0.227 ± 0.123 | 0.241 ± 0.111 | 0.047 | 0.217 | 0.005 | 0.054 |
| **Flavonifractor** | 0.359 ± 0.301 | 0.171 ± 0.293 | 0.209 | 0.148 | 0.029 | 0.194 |
| **Similar changes** | 0.253 ± 0.183 | 0.137 ± 0.132 | 0.003 | 0.148 | 0.003 | 0.040 |
| **Clostridiales** | 0.985 ± 0.926 | 0.682 ± 0.962 | 0.026 | 0.148 | 0.011 | 0.118 |
| **Erysipelotrichaceae UCG 003** | 1.051 ± 0.666 | 0.774 ± 0.654 | 0.026 | 0.148 | 0.032 | 0.194 |

Values are means ± standard deviation *Genus significantly modified after 17 days of treatment were identified using Wilcoxon paired test. P-values were adjusted to control the false discovery rate for multiple testing according to the Benjamini and Hochberg procedure (q value).

inulin group) but ethanol consumption was comparable between the two groups. Inulin had no significant effect on food-related-energy intake that increased in both groups during the withdrawal period (by 61% in placebo group and by 34% in inulin group; Table S3).

Protein and fat intakes (in grams per day) significantly increased by 24% and 50%, respectively, in the placebo group, whereas those changes were not significant in the inulin group (Table S3). The detailed fat intake is presented in Table S4. Subjects in the inulin group consumed significantly less monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) than patients in the placebo group when expressed in g/d (Model 1). Only the effect on PUFAs was maintained when we adjusted for the quantity of ethanol consumed during the second week of the program (Model 2; Table S4). When the results were expressed in % of fatty acids, the intake of saturated fatty acids (SFA) increased significantly in the inulin group compared to placebo while MUFA intake decreased regardless of the model considered for the analysis (Table S4).

We have already shown in a previous study that fiber intake was very low in actively drinking AUD patients. During the second week of the program, at home, the two groups increased significantly their TDF intake to reach, on average, 19 g per day (Table S3). Twenty-eight percent of patients in the placebo group had an intake of more than 25 g/d during the second week of the program (Figure S2 A-B). The supplementation with 8 g of inulin was not sufficient to reach the recommended 25 g of fiber per day since only 38% of the patients in the inulin group had an intake higher than 25 g when the supplementation was taken into account (Figure S2B).

Fructan, FOS and GOS intakes (in g/d) increased significantly in the placebo group and fructan and FOS intakes tend to be lower in the inulin group than in placebo ($\beta = -0.97, p = .08$ and $\beta = -0.76, p = .06$, respectively, in model 2; Table S3). This observation is consistent with the fact that inulin-treated patients had lower fructan and FOS intakes compared to placebo patients. The relative abundance of gut bacteria at the genus level was compared using the Wilcoxon signed rank test followed by Benjamini and Hochberg correction for multiple testing (Table S2).
AUD patients ate less roots and tubers than the placebo (Table S2). Taking into account the inulin supplementation, the total amount of fructan intake averaged 9.4 g per day (1.4 g from food and 8 g from supplementation) in the inulin group and 2.1 g per day in the placebo group during the second week of the program.

The effect of inulin supplementation on micronutrient intakes is presented in supplementary table S5. Zinc was differentially modulated by inulin supplementation when we take into account the gender and the quantity of ethanol consumed during the second week of the program (Model 2). Indeed, zinc intake decreased significantly in the inulin group (β = −2.7, p = .03, Table S5). Inulin supplementation had no impact on the intake of other micronutrients.

**Biological outcomes, except BDNF, are not modulated by inulin supplementation**

It has been shown that inulin supplementation could affect lipid and glucose homeostasis.30,31 We did not observe any difference between the placebo and the inulin group concerning the change (T2-T1) in plasma levels of glucose, cholesterol, triglycerides, and non-esterified fatty acids (Table 3).

Gut hormones are known to be regulated by DF intake including inulin.32 Moreover, it has been shown that AUD patients display altered levels of some gut hormones that could be related to psychological symptoms.33–35 We therefore investigated the effect of inulin on the levels of gut hormones and gut peptides. Inulin supplementation did not affect the levels of gut peptides and gut hormones (Table 3). We did not observe any effect of prebiotic treatment on glucose metabolism as glucagon levels were not modified by the supplementation (Table 3).

We next studied the effect of inulin supplementation on BDNF, a neurotrophic factor that has been associated with various neuropsychiatric disorders.36 Inulin increased the serum BDNF level (Model 2: β = 12.7, p = .04; Table 3). Negative correlations between the BDNF level at T2 and the alcohol craving score (presented below)

### Table 3. Effect of inulin supplementation on biological parameters.

| Metabolism          | Placebo  | Inulin   | Difference in change from baseline M1 | Difference in change from baseline M2 |
|---------------------|----------|----------|---------------------------------------|---------------------------------------|
|                     | T1       | T2       | β [95% CI]               | β [95% CI]               |
| Glucagon (pg/mL)    | 21.5 ± 11.0 | 17.1 ± 6.7 | 23.8 ± 11.2 | 18.9 ± 8.3 | 2.44 [−1.95; 6.83] | 1.67 [−5.22; 5.87] |
| Glucose (mg/dL)     | 73.6 ± 8.2 | 71.7 ± 9.5 | 75.2 ± 12.1 | 74.0 ± 9.2 | 2.13 [−2.92; 7.18] | 1.96 [−3.16; 7.08] |
| Cholesterol (mg/dL) | 4.8 ± 0.9  | 4.5 ± 0.8  | 5.2 ± 1.1  | 4.6 ± 1.0  | −0.14 [−0.55; 0.28] | −0.17 [−0.57; 0.24] |
| Triglycerides (mg/dL) | 1.4 ± 1.2 | 1.1 ± 0.4  | 1.5 ± 1.1  | 1.4 ± 0.9  | 0.31 [−0.01; 0.63] | 0.26 [−0.03; 0.56] |
| NEFA (μmol/L)       | 0.8 ± 0.5  | 0.5 ± 0.2  | 0.9 ± 0.5  | 0.7 ± 0.5  | 0.13 [−0.11; 0.38] | 0.08 [−0.16; 0.31] |

**Gut Hormones/peptides**

| Active GLP-1 (pM) | 0.69 ± 0.57 | 0.38 ± 0.27* | 0.65 ± 0.39 | 0.57 ± 0.47 | 0.11 [−0.12; 0.35] | 0.12 [−0.12; 0.36] |
| GLP-1 (pM)       | 29.2 ± 15.6 | 23.6 ± 11.8 | 28.6 ± 13.6 | 26.0 ± 11.1 | −0.34 [−6.96; 6.3] | −1.58 [−7.81; 4.64] |
| Active Ghrelin (pg/ml) | 189.1 ± 124.5 | 152.2 ± 73.2 | 206.6 ± 143.6 | 228.8 ± 172.1 | 49.0 [−16.59; 114.58] | 46.40 [−20.5; 113.3] |
| Ghrelin total (pg/ml) | 594.0 ± 367.8 | 499.7 ± 235.1 | 621.3 ± 346.3 | 564.2 ± 333.4 | 14.90 [−145.3; 175.1] | 16.79 [−147.1; 180.71] |
| Leptin/BMI        | 544.2 ± 545.6 | 490.3 ± 427.9 | 861.4 ± 506.3 | 722.9 ± 476.2* | 21.43 [−142.7; 185.6] | 26.66 [−149.8; 155.2] |
| PY (pg/ml)        | 94.4 ± 61.6  | 80.2 ± 35.7 | 79.2 ± 32.2 | 71.6 ± 28.7 | −5.19 [−26.8; 16.4] | −7.73 [−29.07; 13.62] |

**Growth factors**

| BDNF (pg/ml) | 41.29 ± 26.47 | 39.9 ± 17.6 | 44.7 ± 24.2 | 51.3 ± 17.8 | 10.32 [−2.2; 22.9] | 12.72 [0.89; 24.54] |

***p < 0.001, **p < 0.01, *p < 0.05 paired T-test or Wilcoxon test: intra-group comparison. β: regression coefficient. M1: model 1 adjusted for gender and the parameter at baseline. M2: model 2 adjusted for gender, the parameter at baseline and the quantity of ethanol consumed during the second week of the program.
were observed in the global population and in the inulin group (r = −0.37, p = .02 in global AUD population and r = −0.67, p < .001 in inulin group; data not shown).

**Inulin can modulate social behavior but does not impact mood and alcohol craving in AUD patients**

Figure 4 shows the evolution of psychological symptoms between baseline and the end of the study. Depression, anxiety, and alcohol craving scores decreased during alcohol withdrawal regardless of the group. The linear regression models revealed that there were no differences in changes from baseline between the placebo and the inulin group for depression, anxiety and craving (Table 4). While the sociability score remained stable during alcohol withdrawal in the placebo group, the patients in the inulin group had an increase in the medium pleasant social activity score (p < .05) which remained significant after adjustment for potential confounders (β = 0.68, p = .039 in model 1 and β = 0.71, p = .03 in model 2; Table 4). A significant positive correlation was observed between the change of *Bifidobacterium* and the change of sociability score (Figure 3f). Inulin supplementation had no effect on fatigue (Table S6).

**Discussion**

The aim of the present study was to promote dietary fiber intake, prone to modulate the gut microbiota in AUD patients, by an intervention of inulin versus placebo performed during alcohol withdrawal period. Indeed, from our previous studies, we know that AUD patients are characterized by gut microbial dysbiosis, and, among nutritional disorders, by an intake of DF below the recommendation of the European Food Safety Authority and of the Belgian Health Council (25–30 g per day for health).19,20

The results obtained with the food survey, carried out during the second week of withdrawal, showed that 8 g of inulin were not sufficient to reach the recommended 25 g/day, which reinforces the coherence of the study design that gradually
increased the amount of inulin up to 16 g per day. Sixteen grams of inulin supplementation were achieved without significant gastrointestinal side effects. Indeed we showed that inulin was well tolerated by AUD patients with no significant differences compared with placebo concerning abdominal pain, bloating or stool frequency. It has been shown in healthy individuals that inulin increased softening of feces and flatulence episodes.28,37 In our study, AUD patients supplemented with inulin had a mean Bristol score between 4 and 5 at day 18, which corresponds to a normal score.38 Flatulence episodes were not measured in our study.

Seventeen days of inulin supplementation lead to selective modifications of the gut microbiota in AUD patients. First, we observed a decreased in α-diversity in inulin subjects compared to placebo. While several observational studies showed a positive correlation between dietary fiber intake and microbial diversity,-

\[ \text{Difference in change from baseline M1} \beta [95\% CI] \]

| Depression                      | Placebo    | Inulin    | Placebo    | Inulin    |
|---------------------------------|------------|-----------|------------|-----------|
| Beck depression inventory       | 22.0 ± 10.7| 10.6 ± 9.2***| 29.0 ± 12.5| 18.0 ± 10.0***|
| BDI suicide score               | 1.1 ± 1.0  | 0.5 ± 0.9  | 1.6 ± 1.4  | 0.9 ± 1.0** |
| BDI fatigue score               | 2.6 ± 1.4  | 1.0 ± 1.3***| 3.4 ± 13.2 | 1.6 ± 1.6***|

| Anxiety                         | Placebo    | Inulin    | Placebo    | Inulin    |
|---------------------------------|------------|-----------|------------|-----------|
| STAI-State                      | 46.0 ± 13.9| 38.1 ± 13.5*| 47.2 ± 15.9| 42.4 ± 13.6*|

| Craving                         | Placebo    | Inulin    | Placebo    | Inulin    |
|---------------------------------|------------|-----------|------------|-----------|
| OCDS                            | 24.3 ± 6.4 | 7.2 ± 6.5***| 25.1 ± 5.2| 9.8 ± 5.3***|
| OCDS OT                         | 10.6 ± 3.74| 4.4 ± 4.4***| 10.6 ± 3.9| 5.7 ± 3.6***|
| OCDS CT                         | 13.7 ± 3.1 | 2.8 ± 2.3***| 14.5 ± 2.4| 4.0 ± 2.3***|

| Sociability                      | Placebo    | Inulin    | Placebo    | Inulin    |
|---------------------------------|------------|-----------|------------|-----------|
| Social high pleasant            | 3.8 ± 1.5  | 4.2 ± 1.4  | 3.8 ± 1.4  | 4.1 ± 1.2  |
| Social medium pleasant score    | 3.4 ± 1.5  | 3.7 ± 1.7  | 3.9 ± 1.3  | 4.4 ± 1.0* |
| Social low pleasant             | 3.0 ± 1.42 | 4.0 ± 1.3**| 3.2 ± 1.5  | 4.0 ± 1.4***|

Values are means ± standard deviation. β: regression coefficient.
M1: Linear regression model adjusted for gender and the parameter at baseline
M2: Linear regression model adjusted for gender, the parameter at baseline and the quantity of ethanol consumed during the second week of the program.
***p < 0.001; **p < 0.01; *p < 0.05 paired T-test or Wilcoxon test: intra-group comparison
AUD, alcohol use disorder; BDI, Beck Depression Inventory; CT: Compulsive Thoughts; OCDS, Obsessive compulsive drinking scale; OT, Obsessive Thoughts; STAI: State-trait anxiety inventory.

By contrast, a small number of studies have reported a decrease in species richness with inulin supplementation.42,43 In our study, we also found a decrease in microbial diversity upon inulin exposure in AUD patients. We can conclude that supplementation with only one type of fiber in AUD patients with a poorly varied diet induces a loss of diversity. Since dietary diversity has been shown to correlate positively with microbiota diversity,44 it is likely that long-term adherence to a varied diet is more important in determining microbial diversity than supplementation with an isolated nutrient for a short period. Future long-term studies should be conducted with a combination of different fibers to expect a beneficial effect on microbial diversity.43 We also observed a significant increase in the relative abundance of Actinobacteria, Bifidobacteriaceae and Bifidobacterium, especially B. adolescentis and B. longum. The other Bifidobacterium species were either absent or marginally present, in our AUD population. Bacteroidaceae and Bacteroides decreased significantly after 17 days of inulin supplementation. These results are in line with a recent systematic
review that highlights a modification of these two genera with inulin supplementation in human studies, and is in accordance with our previous data of intervention study performed in obese patients. As Healey et al, upon inulin intervention, we found a trend toward an increase in Faecalibacterium and a significant decrease in Dorea. Ruminococcus torque was decreased with inulin supplementation in our study. This bacterium, known to be more abundant in intestinal bowel disease (IBD) patients, is a potent mucus degrader and has been associated with a decrease in gut barrier integrity in previous studies. In the placebo group, we observed changes but none of them reached the q value (q > 0.10) meaning that 17 days of abstinence alone was not able to induce strong alterations of the gut microbiota composition. This is in line with our previous work showing a relative stability of the gut microbiota after 3 weeks of withdrawal.

Inulin supplementation had no strong impact on nutrient intake. This is probably due to the duration of the supplementation which was barely 1 week at the time of the nutritional survey. Abstinence alone induces an increase of all macronutrients regardless of the group. However, patients in placebo group increased their fructans, FOS and GOS intake while there were no significant changes in inulin group. Subjects in the placebo group increased their consumption of roots and tuber and bread during week 2, which could explain this result. Patients in the placebo group significantly increased their lipid intake without an increase in a specific type of lipid when looking at the intake as a percentage of total FA.

We also observed a decrease in zinc intake in the inulin group, which could be explained by a decrease in meat intake in the inulin group even if this later result was not significant.

How probiotics might affect food preferences is still unclear but it has been hypothesized that DF with prebiotic properties could act on the microbiota causing the growth of some specific bacteria, which can in turn affect eating behavior. Indeed, it has been suggested that bacteria are submitted to selective and evolutionary pressure and are therefore capable of inducing preferences for certain foods to promote their own growth. Daud et al. found that oligofructose supplementation had an impact on the desire to eat fatty, sweet and salty foods in overweight and obese population. We showed the same effects on food-related behavior upon an inulin-rich diet intervention for 2 weeks in healthy volunteers. It is well known that AUD patients have a craving for sweetie food during the withdrawal. However, in the present study, inulin supplementation did not reveal any impact on sweet intake in AUD patients since we observed an increase in the consumption of sweets and soda in both groups of patients. It is likely that inulin supplementation or the duration of the supplementation are not sufficient to counteract disturbances in the sensory and reward systems that control both alcohol and palatable food craving.

We hypothesized that the altered gut–microbiota–brain axis in AUD patients can be improved by modulating the gut microbiota composition with inulin known to promote beneficial bacteria, like Bifidobacteria. The scores of depression, anxiety, and craving decreased significantly in the two groups of treatment. We did not observe any additional effect of inulin supplementation. It is well known that ethanol has a direct effect on the brain and on negative reinforcement processes. Therefore, stopping alcohol has a beneficial effect on negative emotions, but we have previously shown that the recovery could also be affected by gut dysbiosis. Although inulin increased the level of Bifidobacterium, it was not associated with further improvement in psychological symptoms. No study has investigated yet the effect of inulin on cognitive symptoms or mental health of AUD patients, but it has been studied in other contexts. Smith and colleagues highlighted that the acute administration of 5 g of oligofructose-enriched inulin improved wellbeing and episodic memory in healthy volunteers. In obese patients, 3 months of inulin supplementation improved emotional competence and cognitive flexibility. A recent study in patients suffering from coronary artery diseases has shown that the co-supplementation with 15 g of inulin and Lactobacillus rhamnosus GG during 2 months decreased significantly depression and anxiety scores as well as lipopoly-saccharide and inflammatory markers.
Interestingly, the subjects had an increase in serum BDNF levels when supplemented with inulin compared to placebo. An impact of prebiotics on BDNF levels has already been demonstrated in several mouse studies.\textsuperscript{60,61} BDNF is an important neurotrophin involved in brain plasticity, the levels of which are decreased in anxiety and depression\textsuperscript{62} and preclinical studies have highlighted that the gut microbiota could directly modulate the brain expression of BDNF.\textsuperscript{63,64} Even though we did not measure BDNF levels in the brain, it has been shown that peripheral blood BDNF levels are positively correlated with BDNF in the brain.\textsuperscript{65,66}

Despite the change in BDNF, we did not observe further positive effects of inulin as compared to placebo on psychological symptoms in our AUD population. This can be attributed to several factors. First, the duration of the supplementation, 17 days of supplementation may not be sufficient to observe a significant effect on behavior. Furthermore, in this study, two factors likely modulate the behavior of the patients: inulin supplementation and alcohol withdrawal. We have seen that abstinence alone has a strong impact on depressive symptoms, anxiety and craving, and therefore could mask an additional positive effect of prebiotics. It is also possible that the sample size was not large enough to observe a significant effect of inulin on psychological symptoms, as the study was designed to observe a bifidogenic effect. Other studies with a larger sample size are needed to confirm these results.

However, we observed an increase in the sociability subscore (social medium pleasant score) in the inulin group compared to placebo. A 6-week randomized, double-blind placebo-controlled study demonstrated that a combination of Bimuno\textsuperscript{\textregistered} galactooligosaccharide with a casein/gluten-free diet, which increased \textit{B. longum}, improved behavioral symptoms including sociability score in autistic children.\textsuperscript{67} Interestingly, in our study the improvement of the sociability score was correlated with the increased \textit{Bifidobacterium} level. The link between the gut microbiota and sociability has been demonstrated in preclinical studies\textsuperscript{15} and we have previously shown a link between leaky gut and social impairment in AUD patients.\textsuperscript{16} It remains unclear how microbial changes may induce some of the behavioral effects, but it has been shown that \textit{Bifidobacterium longum} NCC3001 restores anxiety-like behavior through the vagus nerve in mice.\textsuperscript{63} However, our results should be interpreted with caution as only one out of the six sociability sub-scores was significantly modified by inulin supplementation.

One of the limitations of our study that may have hidden changes related to the prebiotic intervention, was the higher severity of the AUD DSM-5 scores observed at baseline in the treatment group as well as the higher proportion of females. Gender is known to influence the biological parameters but also the recovery from psychological symptoms during abstinence that are usually less rapid in female than male patients.\textsuperscript{68} Furthermore, almost half of the patients relapsed during the intermediate week. This parameter may also modify the trajectory of symptom recovery as well as changes in the gut microbiota composition. It would have been interesting to stratify the population according to these two parameters that was impossible with our small sample size. However, fitting linear models on these two variables limited bias. Finally, the time point of the dietary data collection did not match perfectly with the fecal sample collection at T2. This makes it more difficult to interpret microbial changes in relation to nutritional intake.

In conclusion, our pilot work is the first showing that inulin supplementation is able to modulate the gut microbiota of AUD patients, although it had only a limited impact on biological outcomes or mental health. Inulin supplementation did not promote the expected effects on depression, anxiety and craving probably due to 1) the small sample size, 2) the short duration of supplementation, and 3) the fact that alcohol withdrawal already has a strong impact on psychological symptoms. However, we have shown that bacteria modulated with inulin supplementation could potentially be involved in sociability. Other studies involving longer treatment and larger sample size are needed to investigate whether inulin could be an appropriate nutritional approach to improve psychological symptoms and the biological outcomes of patients with alcohol use disorder.
Methods

Study design

This randomized, double-blind, placebo-controlled study was conducted from October 2018 to December 2019. Each subject was randomly assigned to daily intake of inulin (Inulin group) or maltodextrin (Placebo group) using the method of randomly permuted blocks (50 subjects randomized into 5 blocks). The randomization was performed via the website http://www.randomization.com by a person not involved in the study in order to ensure the double blind.

Compliance was assessed by counting the bags that were returned by subjects. Participants with a compliance of less than 80% were considered to be non-compliant.

Participant selection

A total of 50 AUD patients hospitalized for a 3-week highly standardized alcohol-detoxification program in St-Luc academic hospital, Brussels, Belgium, were enrolled on voluntary basis. This program consists of 2 weeks at the hospital (weeks 1 and 3), separated by 1-week outpatient care (week 2). The severity of AUD was checked by a psychiatrist using the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5).

Inclusion criteria were as follows: male or female, 18–65 years old, French speaking, and active alcohol consumption until at least 48 hours prior to admission. Patients suffering from another addiction (except tobacco), with inflammatory bowel disease or other chronic inflammatory diseases (such as rheumatoid arthritis), cancer, metabolic diseases such as obesity (BMI ≥ 30 kg/m2), diabetes, bariatric surgery, and severe cognitive impairment (MMSE < 24) were excluded from the study. Patients with known cirrhosis or significant hepatic fibrosis (≥F2) detected by Fibrosan (>7.6 kPa) at admission were also excluded from the study. Other exclusion criteria were the following: the use of antibiotics, probiotics or prebiotics within 2 months prior to enrollment and the use of non-steroidal anti-inflammatory drugs or glucocorticoids within one month prior to enrollment.

The trial protocol was published on protocols.io (dx.doi.org/10.17504/protocols.io.bvs2n6ge). The study was approved by the institutional ethics committee (N°2017/04JUL/354). All participants signed informed consent prior to inclusion and the trial was registered in the clinicaltrials.gov registry (ClinicalTrials.gov identifier: NCT03803709).

The primary outcome of this trial was the effect of the prebiotic intervention on the gut microbiota composition. The secondary outcomes were the effect of inulin supplementation on gastrointestinal tolerance, nutritional intake, biological markers of satiety, lipid and glucose homeostasis and psychological parameters.

Dietary intervention

Inulin (Fibroline*) and maltodextrin (placebo) were kindly provided in similar opaque packaging by Cosucra (Warcoing, Belgium) to ensure the double-blind procedure. The patients were asked to dilute the powder in a hot drink (tea, coffee) or yogurt. According to previous studies on the effect of inulin and knowing that is a digestible non-fermentable carbohydrate, maltodextrin has been selected as placebo. Inulin and maltodextrin had the same taste, odor and texture.

In order to reduce the gastrointestinal side effects, the dose of inulin or maltodextrin increased gradually from 4 to 16 g per day during the treatment (4 g from Day 3 to Day 4; 8 g from Day 5 to Day 14 and 16 g from Day 15 to Day 19 of the detoxification program). Indeed we have previously shown that 16 g of inulin per day was well tolerated and had a bifidogenic effect in obese patients.

Outcomes

Gut microbiota composition

Stool samples were collected at Day 2 (T1) and at the end of the intervention (Day 19 – T2). They were collected in a sterile container and stored immediately at −20°C and then transferred to −80°C within 5 to 10 hours. Genomic DNA was extracted from the feces using a QIAamp DNA Stool Mini Kit (Qiagen, Germany), including a bead-beating step and following the protocol Q. After extraction, dsDNA concentration was measured using the NanoPhotometer®
Spectrophotometer (Implen, CA, USA). The composition of the gut microbiota was analyzed by Illumina sequencing of the 16S rRNA gene. The V3-V4 region of the 16S rRNA gene was PCR-enriched using the primer pairs V3F_Nextera (CCTACGGAGGCGAGCAG) and Meta_V4_806 R (GGACTACHVGGGTWTCTAAAT). The amplicons were purified, quantified and sequenced using an Illumina MiSeq to produce 2x300-bp sequencing products at the University of Minnesota Genomics Center. During the sequencing run, a quality score is assigned to each base call, using the Illumina’s quality scoring methodology. The mean quality score for each sample was > 33.8. Then, the sequence reads are converted automatically to FASTQ using a bc1fastq converter. 16S rDNA amplicon sequences were analyzed using FROGS pipeline.\(^7\) Amplicons were filtered according to their size then clustered into OTUs using Swarm (aggregation parameter d = 1 + d = 3). Chimera were removed using VSEARCH combined with an innovative chimera cross-validation and OTUs were kept when representing more than 0.005% of the total number of sequences.\(^7\) OTUs were classified using the reference database Silva138 16S with a pintail quality of 100.\(^7\) Relative abundance of each OTU was calculated after data normalization using a threshold of 33133 reads per sample.

qPCR of 16S rDNA was used to quantify the abundance of total bacteria (F: ACT-CCT-ACG-GGA-GGC-AGC-AG, R: ATT-ACC-GGC-GCT-GTG) and Bifidobacterium spp (F: GAT-TCT-GGC-TCA-GGA-TGA-ACG-C, R:CTG-ATA-GGA-CGC-GAC-CCC-AT). PCR amplification was carried out as follows: 10 min at 95°C, followed by 45 cycles of 3 s at 95°C, 26 s at 58°C or 60°C, and 10 s at 72°C. Detection was achieved with the QuantStudio3 instrument and software (Applied Biosystems) using the GoTaq qPCR MasterMix Plus for SYBR Assay (Promega). BSA was added to samples. Each assay was performed in duplicate in the same run. For construction of standard curves, fivefold dilution series from target species genomic DNA preparations (DSMZ, Braunschweig, Germany) were applied to the PCR.

**Gastrointestinal tolerance**

Gastrointestinal symptoms were measured using a French version of a self-reported questionnaire initially used to evaluate the symptoms of irritable bowel syndrome developed by gastroenterologists at the St-Luc hospital as described before.\(^18,73\) Patients completed this questionnaire at baseline and then every other day after the beginning of supplementation.

**Dietary intake**

On Day 2 of alcohol withdrawal, all participants were interviewed using three nonconsecutive 24-h dietary recall (related to the week before hospitalization: week 0) by a trained dietician as previously described.\(^18\) During the second week of the program (week 2: at home) patients were asked to complete a food diary in which they registered all the food and drinks consumed during 3 defined days (two weekdays and one weekend day). The participants were instructed to specify all ingredients per eating moment: breakfast, morning snack, lunch, afternoon snack, dinner, and evening snack. Detailed guidance notes, including ingredients most often omitted (e.g. fats, added sugars, beverages) and their unit of measurement (weight and household units), were provided in the diary. To avoid bias, participants did not receive any advice from the dietician regarding their eating habits. Advice was provided “on demand” at the end of study. At the beginning of week 3, careful analysis of the food diary was performed by the dietician during a face-to-face interview with the patient. Energy and nutrient intakes were evaluated using the Nubel Pro program (Nubel asbl, Belgium) and the French food composition database (CQUAL 2017). Dietary fibers including soluble fibers, insoluble fibers, fructans, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) were evaluated using a specific database from the FiberTAG project.\(^7\) The results were expressed in quantities and in proportion of total energy intake (EI). The lipid intakes were also expressed in proportion of total fatty acids (FA).

**Blood parameters**

Fasting blood samples were collected at T1 and T2. Blood samples were centrifuged at 1000 g for 15 min at 4°C and the plasma was frozen at –80°C in a biobank. Plasma concentrations of gut hormones (GLP-1, leptin, ghrelin and PYY) and
growth factors (Brain derived neurotrophic factor [BDNF]) were determined using the Meso Scale Discovery (MSD) U-PLEX assay (Rockville, MD, USA). Plasma triglycerides, total cholesterol, and glucose were dosed by enzymatic colorimetric test (Diasys Diagnostic and System, Holzheim, Germany). Plasma non-esterified fatty acid levels were assessed using a commercially available enzymatic assay (Randox Laboratories, Crumlin, UK).

**Psychological symptoms assessment**

At baseline (T1: day 1–2) and at the end of the supplementation (T2: day 19) all patients were tested for anxiety, depression and alcohol craving with self-reported questionnaires (French versions): the State-Trait Anxiety Inventory [STAI form YA], the Beck Depression Inventory [BDI] and the Obsessive-Compulsive Drinking Scale modified version [OCDS] as described previously. The OCDS can be divided into two subscales, an ‘obsessive’ subscale and a ‘compulsive’ subscale. We used a modified version adapted to withdrawal that excluded items related to drinking. Fatigue was assessed using the Multidimensional Fatigue Inventory-20 and sociability using the social situation test. All these questionnaires have been described previously.

**Statistical analysis**

Statistical analyses were performed using SAS version 9.4, R studio version 3.5.1 and Graphpad Prism 8.0.

Data were presented as mean ± standard error of the mean (SEM) or mean ± standard error deviation (SD). Normality was assessed by the Shapiro–Wilk test. According to data distribution, Mann–Whitney U-test or t-test were performed to compare the baseline characteristics of placebo and inulin groups.

We calculated the total dietary fiber intake for each patient and we added the 8 g of inulin in the inulin group at week 2. The evolution between T1 and week 2 in each group was assessed using a paired t-test or a Wilcoxon signed-rank test. Then we calculated the proportion of patients who achieved a fiber intake of at least 25 g per day using a Fisher test.

The effect of inulin supplementation on gastrointestinal symptoms was studied using a linear mixed model with time and treatment as fixed effects and patient as random effect. A second model adjusting for gender was performed. The gastrointestinal scores at day 1 and day 18 were then compared in each group in order to study the evolution of the symptoms between baseline and the end of the supplementation. To do so, we used paired t-test or Wilcoxon signed-rank test. Finally, the changes from baseline (D18-D1) were compared between groups using the Mann–Whitney U test or t-test.

For gut microbiota analysis, phyla, families and genus with an average relative abundance superior to 0.1% were analyzed. We used a Mann–Whitney U test in R to compare the relative abundance between the placebo and inulin group and the within group analyses were evaluated using a Wilcoxon paired test. The p-values were adjusted to control for the false discovery rate for multiple testing according to the Benjamini and Hochberg procedure. q < 0.10 was considered statistically significant.

For each psychological and biological outcome, a change variable was calculated as the difference between end-of-study (T2) and baseline measurements (T1). For nutritional data the changes variables were calculated as the difference between week 2 and week 0. As there was an imbalance between genders (24% of women in placebo group vs 50% in the inulin group) and knowing that gender can influence the evolution of psychological symptoms, we adjusted the linear regression models for gender and/or alcohol consumption to avoid potential bias. In order to study the effect of the withdrawal period alone, within group analyses were evaluated using a Wilcoxon signed-rank test. P values <.05 were considered statistically significant.

Sample size was estimated using G*Power based on the bifidogenic effect of inulin. Therefore, we estimated that a total sample size of 50 participants, with a 20% drop out during the study and 20 patients in each group completing the study provides 80% power to observe an effect size of 0.34 for the relative abundance of *Bifidobacterium* genus using a 0.05 two-sided significance level.
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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The accession number for the raw data generated with the 16S rRNA gene sequencing reported in this paper is BioProject PRJNA745947 (SRA) and are available here https://www.ncbi.nlm.nih.gov/bioproject/PRJNA745947/.

Authors’ contributions

Conceptualization & design: SL, NMD, PdT, PS, AMN. Data curation: CA, SL, NMD, AMN, PdT, PS. Formal analysis: CA, VT, SL, VC, QL, NMD, AMN. Funding acquisition: NMD, PdT, PS. Investigation: CA, SL, PS. Methodology: SL, NMD, PdT, PS, AMN, HP, LBB. Project administration: SL, AMN, NMD, PdT, PS. Resources: NMD, PdT, PS. Software: CA, VT, VC. Supervision: NMD, PdT, SL, PS. Validation: SL, NMD, AMN, PdT, PS, LBB. Writing original draft: CA, SL. Writing review & editing: CA, SL, NMD, PdT, PS, AMN, QL, LBB, HP.

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